

AD\_\_\_\_\_

Award Number: W81XWH-05-1-0086

TITLE: Enhanced Androgen Signaling With Androgen Receptor Overexpression in the Osteoblast Lineage Controls Skeletal Turnover, Matrix Quality and Bone Architecture

PRINCIPAL INVESTIGATOR: Kristine M. Wiren, Ph.D.  
Karl Jepsen, Ph.D.

CONTRACTING ORGANIZATION: Oregon Health & Science University  
Portland, Oregon 97239-3098

REPORT DATE: December 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

<b>REPORT DOCUMENTATION PAGE</b>				<i>Form Approved</i> <b>OMB No. 0704-0188</b>	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>					
<b>1. REPORT DATE (DD-MM-YYYY)</b> 01-12-2006		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED (From - To)</b> 15 Nov 2005 – 14 Nov 2006	
<b>4. TITLE AND SUBTITLE</b>  Enhanced Androgen Signaling With Androgen Receptor Overexpression in the Osteoblast Lineage Controls Skeletal Turnover, Matrix Quality and Bone Architecture				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-05-1-0086	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b>  Kristine M. Wiren, Ph.D. and Karl Jepsen, Ph.D.  E-Mail: <a href="mailto:wirenk@ohsu.edu">wirenk@ohsu.edu</a>				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Oregon Health & Science University Portland, Oregon 97239-3098				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> <p>Androgens have been shown to be important mediators of bone growth and remodeling independent of estrogen. We genetically engineered transgenic mice in which androgen receptor (AR) overexpression is skeletally targeted in two separate models to better understand the role of androgen signaling directly in bone. The central hypothesis of this proposal is that AR transactivation in the osteoblast lineage provides key regulatory signals that influence the progression of osteoblast differentiation and osteogenesis, control the resorption of calcified bone, and modulate lineage determination in the marrow, to influence skeletal architecture and matrix quality. In the second year, we have completed the initial analysis of the second line of AR-transgenic mice created using the col2.3 promoter to drive AR expression. AR overexpression in mature osteoblasts results in reduced turnover and inhibition of bone formation at the endosteal surface, resulting in thinner cortical bones with poor biomechanical properties, including a reduction in maximum load, strength and work to failure (Specific Aim 1). Ongoing analysis in both models of AR2.3- and AR3.6-transgenic animals suggests that AR transactivation in mature osteoblasts is primarily responsible for mediating the effects of androgen on matrix quality and/or mineralization (inhibitory), while immature cells mediate effects of androgen on the periosteum and body composition (anabolic). In both AR overexpression models, enhanced androgen signaling during growth produces a low turnover state and may be detrimental to skeletal quality with more damageability. After a sustained hypogonadal period, both males and females demonstrate improved bone mineral, but only males show improvements in body composition including increased lean and reduced fat mass. Thus, results to date indicate androgen is not anabolic in mineralizing bone cells, and raise concerns regarding androgen administration in young adults.</p>					
<b>15. SUBJECT TERMS</b> Androgen, Androgen receptor, Transgenic, Bone mass, Bone quality, Biomechanics					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>  UU	<b>18. NUMBER OF PAGES</b>  68	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U			<b>19b. TELEPHONE NUMBER (include area code)</b>

## Table of Contents

<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>4-17</b>
<b>Key Research Accomplishments.....</b>	<b>17-18</b>
<b>Reportable Outcomes.....</b>	<b>18</b>
<b>Conclusions.....</b>	<b>18-19</b>
<b>References.....</b>	<b>19</b>
<b>Appendices.....</b>	<b>20-68</b>

## Introduction

Androgen deficiency (as a result of aging, hypogonadism, glucocorticoid therapy, or alcoholism), and other behaviors (chronic smoking, malabsorption and bone marrow malignancies) are associated with the development of osteoporosis in men (1). Osteoporosis is also an important and debilitating side effect of androgen deprivation therapy in conjunction with the treatment of prostate cancer (2, 3). At any one time, osteoporosis affects 20 million Americans. Nearly one-quarter of the patients who suffer a hip fracture die within the first year; 50% of patients are unable to walk without assistance; and 33% are totally dependent (4, 5). Of the 1.3 million bone fractures that can be attributed to osteoporosis every year, 150,000 are hip fractures that occur in men with lifetime risk for the development of fracture at nearly 15% (6). In addition, it is also clear that androgens have an important but very much under-appreciated role in women (7). Other health problems may also be affected by androgen action, including atherosclerotic vascular disease, age-related weakness and disability, memory loss, etc. Since osteoporosis is often coupled with a hypogonadal state, developing an understanding of androgen action in the skeleton may provide insight into development of novel therapeutics for the treatment of osteoporosis and metabolic bone disease.

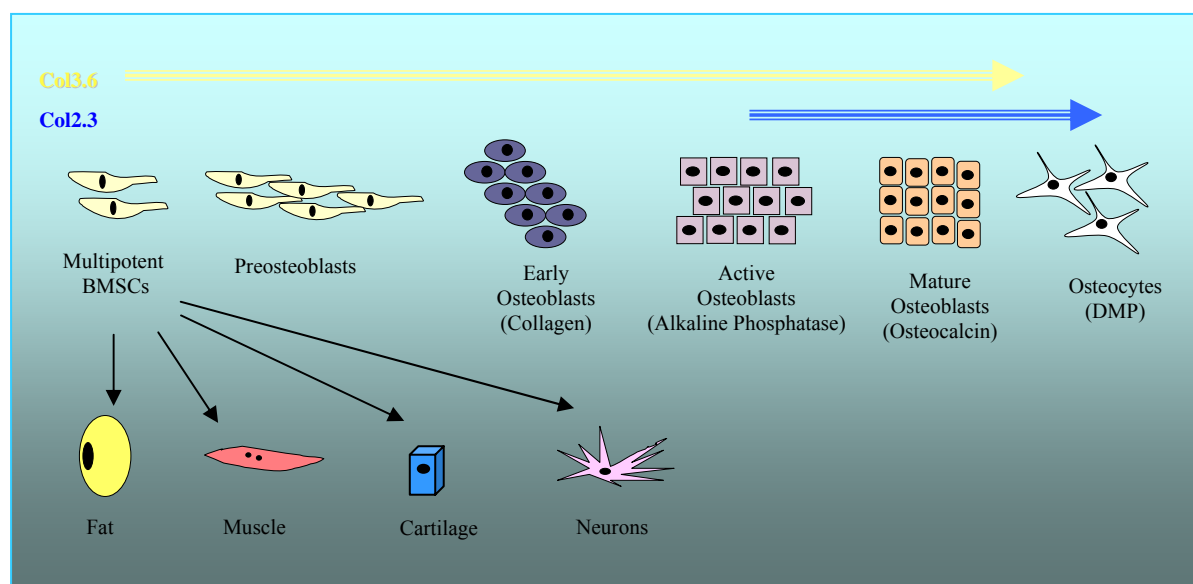
The distinct contribution of androgen to the maintenance of a healthy skeleton remains controversial, since the major androgen metabolite testosterone can serve as the substrate for the production estradiol via aromatase activity. As a consequence, some testosterone action may result from estrogen receptor-dependent activation after conversion to 17- $\beta$  estradiol. Overexpression of androgen receptor (AR), combined with the use of non-aromatizable androgens that cannot serve as a substrate for aromatase conversion (e.g. 5 $\alpha$ -dihydrotestosterone; DHT), should enhance our understanding of the *specific* role for androgen in bone biology. The goal of this program is to gain a comprehensive understanding of the cascade of molecular and cellular events by which androgen signaling influences skeleton homeostasis.

Our proposed studies have substantial military significance. The stated goals of the Bone Health and Military Readiness program are to advance the understanding of methods to improve bone health of young men and women, to enhance military readiness by reducing the incidence of fracture during physically intensive training, and to reduce the incidence of osteoporosis later in life. As little is known about the direct actions of androgens on osteoblasts, our comprehensive approach using unique animal models of enhanced androgen responsiveness with distinct bone-targeted AR-transgenic families, combined with the novel studies of DHT modulation of osteoblast differentiation and osteoblast-osteoclast signaling, will provide insights into normal bone homeostasis. Understanding the consequences of androgen action in bone is particularly important given increased anabolic steroid abuse. In addition, since bone architecture and bone material properties play important roles in stress fracture, analysis of this model represents a unique opportunity to characterize the consequences of androgen action in both genders on bone microarchitectural quality and the integrity of the skeleton *in vivo*.

## Body

In the second year of this grant, we have primarily focused on characterization of a distinct set of AR-transgenic mice, AR2.3-transgenic families, that were constructed using a smaller collagen promoter fragment to drive AR overexpression in mature osteoblasts (as outlined in Specific Aim 1). Thus, in addition to the AR3.6-transgenic families we have previously created with AR overexpression in stromal cells and throughout the osteoblast lineage including mature osteoblasts, the AR2.3-transgenic mice now also provide models for the characterization of enhanced androgen signaling in distinct skeletal compartments (see Fig. 1). Importantly in both

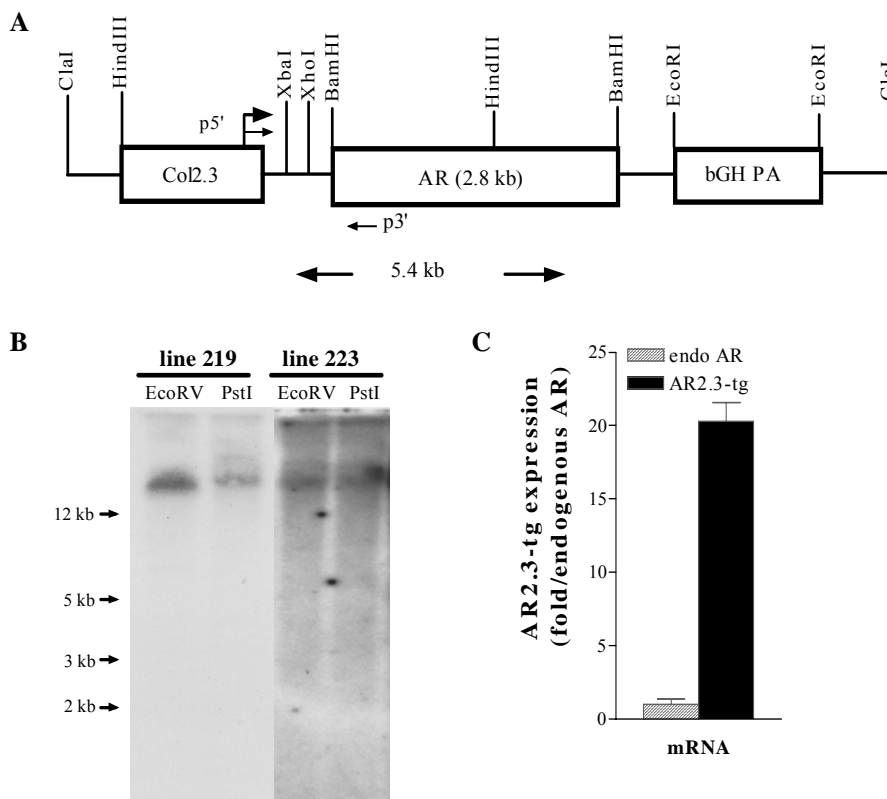
models, enhanced androgen action occurs only in those cells with elevated levels of AR (skeletally-targeted) as a consequence of enhanced AR signaling, without changes in circulating steroid levels and without systemic androgen administration. Because of distinct and overlapping expression profiles as shown in the schematic below, comparison of the skeletal phenotypes characterized in these two models of enhanced androgen action are postulated to aid in the identification of cells within the osteoblast lineage that are most important in mediating a specific response in bone modeling/remodeling characteristics. For example, phenotypes identified as similar in both col2.3 and col3.6 suggests that mature osteoblasts/osteocytes are important mediators of the response, since there is overlap in promoter activity in those cell types. In contrast, phenotypes that are more pronounced in col3.6 than col2.3 suggest that stromal or immature osteoblasts are primary mediators.



Our goal as proposed in Specific Aim 1 is to contrast the skeletal phenotype of AR2.3- with AR3.6-transgenic animals (with different AR overexpression profiles in the osteoblast lineage), in the adult and in the hypogonadal state in both genders, to identify direct androgen actions *in vivo*. This analysis will allow us to test the hypothesis that distinct profiles of AR overexpression in the osteoblast lineage will result in distinct skeletal phenotypes between AR2.3- vs. AR3.6-transgenic mice. The progress report is divided into three sections: 1) completion of analysis of the adult AR2.3-transgenic mouse model (manuscript in preparation); 2) initiation of studies in a low bone turnover situation after gonadectomy in both males and females in both AR2.3- and AR3.6-transgenic mice for evaluation of bone parameters; and 3) analysis of body composition changes in low turnover gonadectomized mice.

**Section 1: Generation of col2.3 AR transgenic mice:** We have recently derived a distinct set of AR-transgenic mice using a shorter fragment of the collagen promoter, the col2.3 promoter, which controls AR-transgene expression only in mature osteoblasts. The full length rat AR cDNA fragment was cloned downstream of the rat 2.3-kb collagen I  $\alpha 1$  promoter and upstream of the bovine growth hormone polyadenylation (bGH-PA) signal (Fig. 1A). The 5.4-kb linear DNA fragment containing the AR2.3 fusion gene was excised from the flanking vector with *Cla*I and used for pronuclear injection of mouse B6D2F1 embryos to generate the AR2.3-transgenic founders. Positive founders were identified by PCR genotyping. For analysis, two independent AR2.3-transgenic lines are being bred to wild type B6D2F1 mice to control for so-called position

effects of the inserted transgene (family 219 and 223). Southern analysis has confirmed a single insertion site for the AR2.3-transgene (Fig. 1B), with approximately 20-fold enrichment of AR transgene expression versus the endogenous AR in calvaria as determined by real-time qRT-PCR quantitation (Fig. 1C).



**Figure 1. Generation of transgenic mice with mature osteoblast-targeted AR overexpression. A.** A schematic representation of the AR2.3-transgene. **B.** Southern analysis for characterization of transgenic animals. Genomic DNA was isolated from both AR2.3-transgenic founder lines (219 and 223), digested with *EcoRV* or *PstI* and subjected to Southern blot analysis after hybridization with the AR2.3-transgene product. Analysis indicates a single insertion site. **C.** Expression of the AR2.3-transgene (AR2.3-tg) relative to the endogenous AR (endo-AR) gene in calvaria was estimated by real-time qRT-PCR analysis.

To further characterize AR2.3-transgenic mice, we determined the pattern of AR-transgene mRNA expression in a wide variety of tissues by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Tissue was harvested from male AR2.3-transgenic mice, total RNA was isolated and gene expression was characterized by qRT-PCR analysis as we previously described (8). As shown in Table 1, AR-transgene expression under the control of the col2.3 promoter results in bone-targeted expression, consistent with other reports (9) and as we have also seen with col3.6 expression patterns in AR3.6-transgenic mice (10). As expected, colAR transgene expression was elevated in bone (calvaria and tibia) with very low levels in all other tissues analyzed including muscle, skin, heart, kidney, fat, liver and tendon. We also determined AR-transgene expression in calvaria by qRT-PCR analysis using calvaria mRNA. Results show AR transgene expression 12 - 20 fold elevated relative to the endogenous AR gene for AR3.6-transgenic and AR2.3-transgenic mice, respectively. This

result suggests similar levels of AR overexpression in both models in calvaria, with lower levels present in long bone (Table 1).

<i>Tissue</i>	<i>Col2.3 AR level</i>	<i>Fold difference</i>
Calvaria	1.0000 $\pm$ 0.2373	n.a.
Tibia	0.0307 $\pm$ 0.0098	-33
Thymus	0.0066 $\pm$ 0.0011	-152
Lung	0.0054 $\pm$ 0.0005	-185
Heart	0.0047 $\pm$ 0.0019	-213
Kidney	0.0044 $\pm$ 0.0006	-227
Fat	0.0027 $\pm$ 0.0007	-370
Spleen	0.0025 $\pm$ 0.0004	-400
Muscle	0.0006 $\pm$ 0.0000	-1667
Skin	0.0006 $\pm$ 0.0002	-1667
Ear	0.0004 $\pm$ 0.0001	-2500
Liver	0.0004 $\pm$ 0.0002	-2500
Tendon	0.0003 $\pm$ 0.0000	-3333
Intestine	0.0000 $\pm$ 0.0000	n.d.

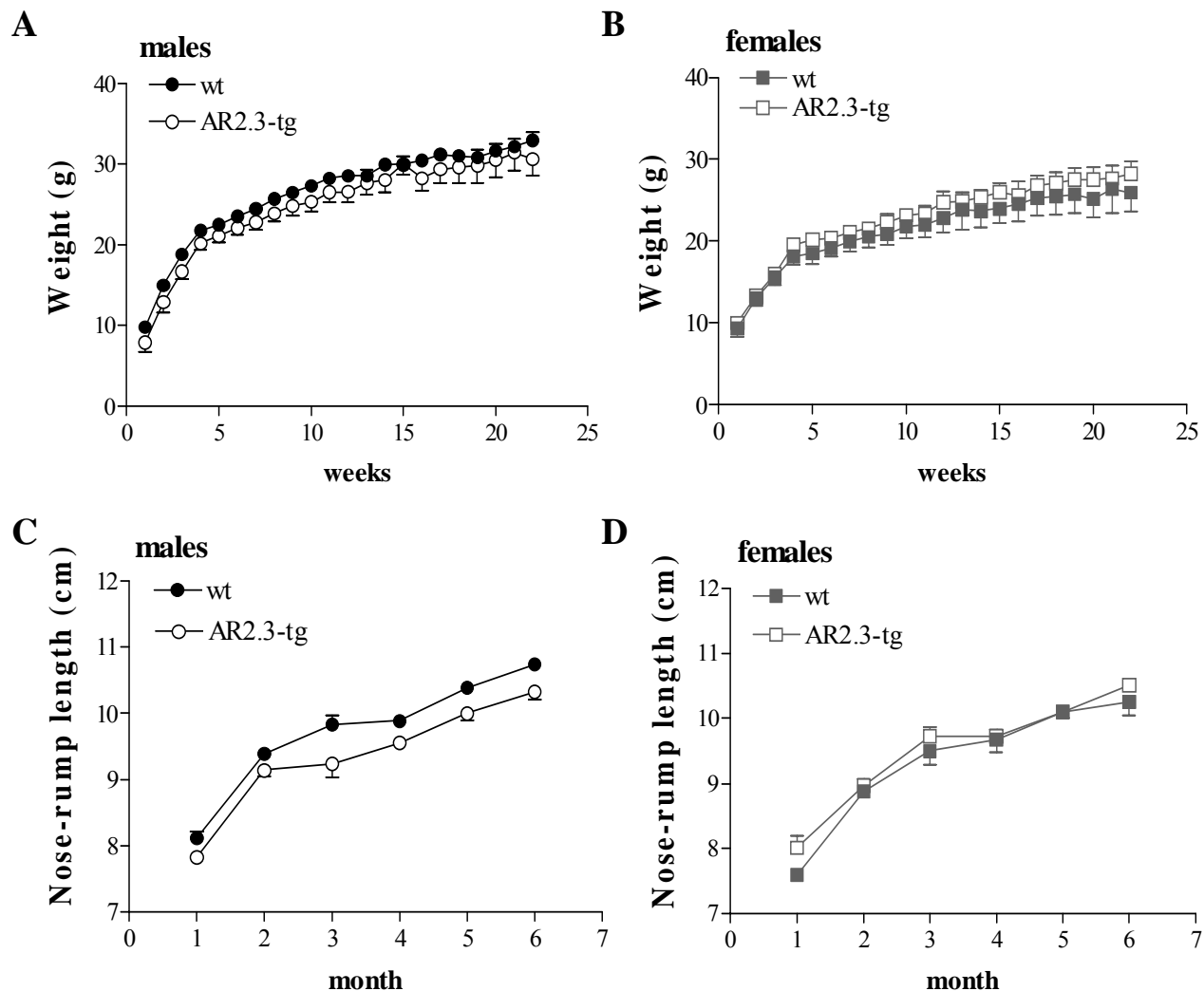
**TABLE 1. Analysis of transgene expression in a variety of tissues from AR2.3-transgenic mice.** Tissues listed were harvested from male AR2.3-transgenic mice and total RNA was isolated (n = 5). Expression of the AR2.3-transgene was evaluated by real-time qRT-PCR analysis after normalization to the total RNA concentration using the RiboGreen assay. Data are expressed relative to the expression level in calvaria as mean  $\pm$  SEM. n.a., not applicable; n.d., not detectable.

*Phenotype in AR2.3-transgenic mice with bone-targeted AR overexpression.* We first determined the effect of bone-targeted AR overexpression on body weight gain and nose-rump length over a 6-month period. At birth animals were indistinguishable and as the mice aged, AR2.3-transgenic males and females gained length and weight similar to wild-type controls (Fig. 2A-D). This result is in contrast to previous analysis with AR3.6-transgenic mice (10), where transgenic males were both significantly shorter and weighed less than wild-type controls.

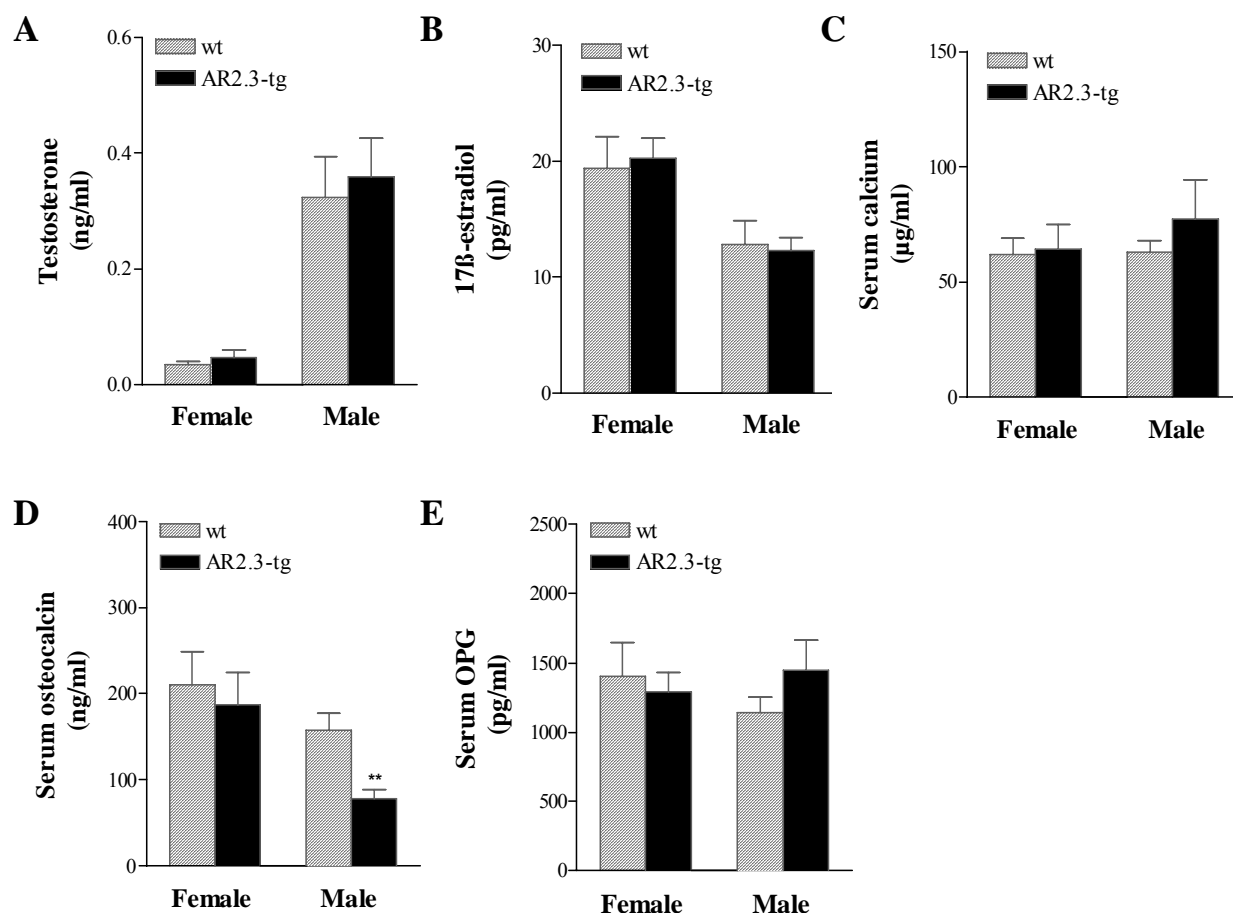
AR2.3-transgenic mice were evaluated for serum biochemistry and hormone levels at 8 weeks of age. As expected, serum testosterone and estradiol levels were not significantly different between littermate controls and AR2.3-transgenic animals in either sex (Fig. 3A, B). There were also no significant differences in serum calcium levels between AR2.3-transgenic and littermate controls (Fig. 3C). Interestingly, there was a significant ~50% decrease in serum osteocalcin levels in male AR2.3-transgenic animals but not in females ( $P < 0.01$ , Fig. 3D). Serum osteoprotegerin (OPG), an important inhibitor of osteoclastogenesis (11), was also analyzed. OPG was modestly but non-significantly increased in AR2.3-transgenic males, with little effect in AR2.3-transgenic females compared to littermate controls ( $P < 0.01$ , Fig. 3E).

Body composition and bone density were then evaluated by dual energy x-ray absorptiometry (DXA) in male and female AR2.3-transgenic mice and littermate wild-type controls at 2 months (Fig. 4). Although areal BMD was not affected (Fig. 4A), bone mineral content (BMC) tended to be reduced in male AR2.3-transgenic mice ( $P = 0.057$ ; Fig. 4B). Because areal BMD is the quotient of BMC and bone area, the decrease BMC is a consequence of reduced mineral, and no effect was noted on BMD because area also decreased in AR2.3-transgenic males. While systemic androgen treatment is known to affect

body compositional changes, no difference was noted in either lean mass or fat mass in either males or females (Fig. 4C, D), indicating skeletal targeting of the AR transgene.



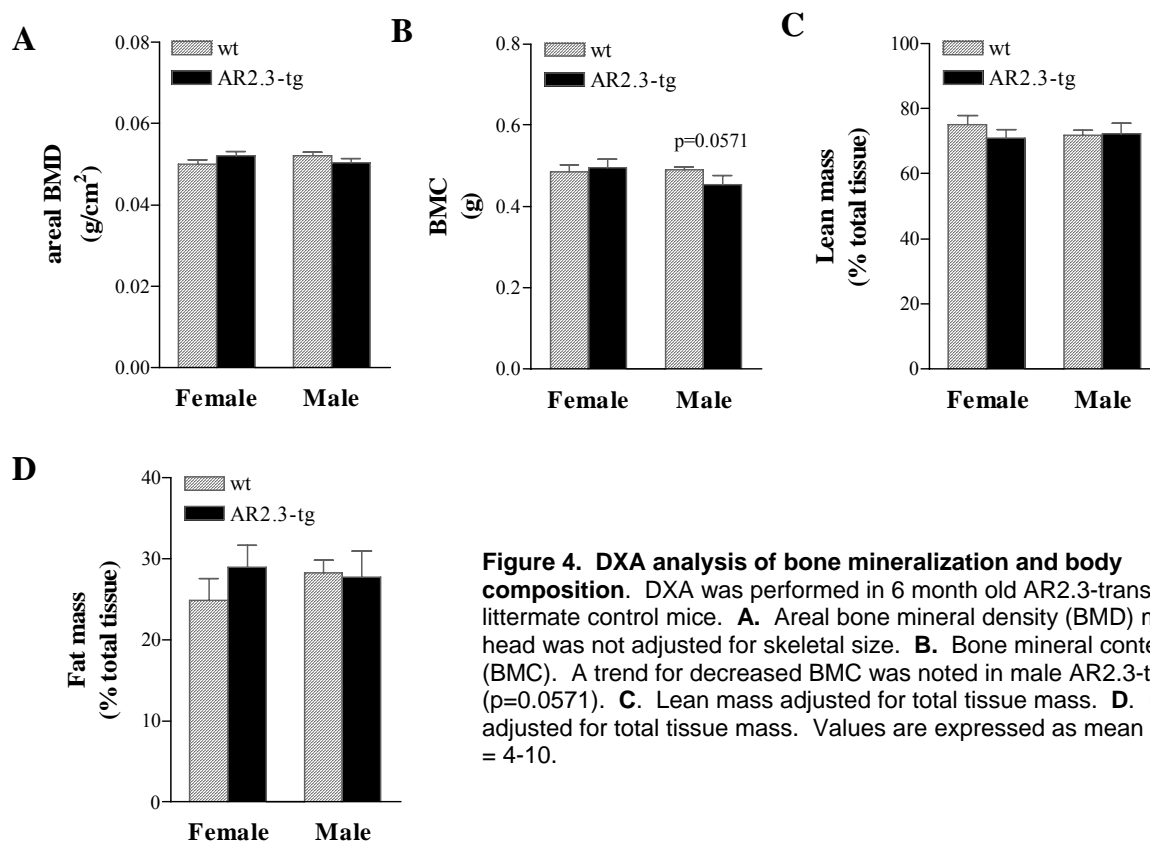
**Figure 2. Age-related changes in body weight and nose-rump length in AR2.3-transgenic mice.** Body weight and nose-rump-length determinations were carried out weekly or monthly, respectively, over six months in both genders in both wild-type (wt) and AR2.3-transgenic (AR2.3-tg) mice ( $n = 4-5$ ). **A.** Weight gain in growing male mice. Analysis for the effects of time and genotype by repeated measures two-way ANOVA revealed an extremely significant effect of time ( $F = 218.36$ ;  $P < 0.0001$ ) but not genotype and with no interaction. **B.** Weight gain in female mice. Similar to male mice, analysis revealed an extremely significant effect of time ( $F = 114.80$ ;  $P < 0.0001$ ) but not genotype and with no interaction. **C.** Nose-rump length in male mice. Analysis revealed an extremely significant effect of time ( $F = 228.54$ ;  $P < 0.0001$ ) and modest effect of genotype ( $F = 15.87$ ;  $P < 0.05$ ) with no interaction. **D.** Nose-rump length in female mice. In contrast to the male mice, analysis revealed no effect of genotype but an extremely significant effect of time ( $F = 149.48$ ;  $P < 0.0001$ ) with no interaction. All data is expressed as mean  $\pm$  SEM.



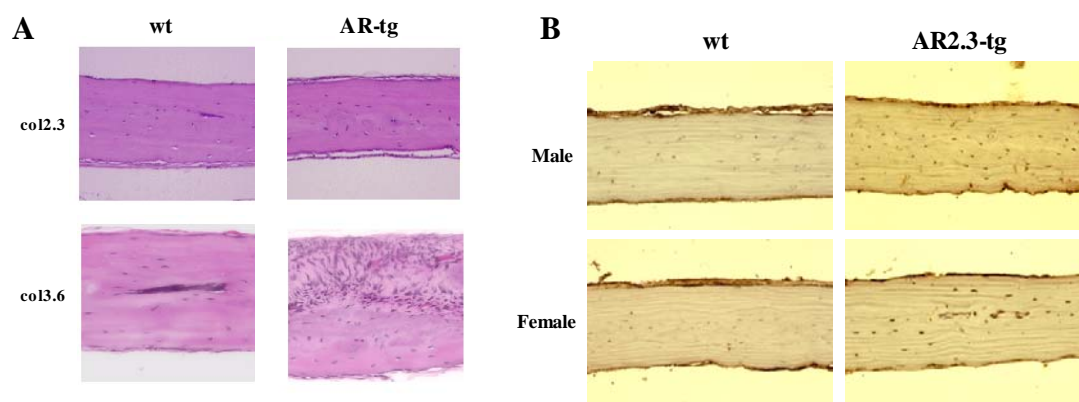
**Figure 3. Biochemical analyses of serum hormone levels and skeletal markers in AR2.3-transgenic animals.** Comparisons were performed between littermate control (wt) and AR2.3-transgenic (AR2.3-tg) animals (n=8-20). Serum from 8-week-old mice was analyzed to determine levels of hormones and markers of calcium metabolism. Assays were performed in duplicate by RIA for 17β-estradiol, and by EIA for testosterone and intact mouse osteocalcin, and for calcium by the colorimetric cresolphthalein-binding method. **A.** Testosterone. **B.** 17β-estradiol. **C.** Calcium. There were no statistical differences between 17β-estradiol, testosterone or calcium levels. **D.** Osteocalcin levels were significantly reduced in male AR2.3-tg mice. **E.** OPG circulating levels were elevated in males but not in female AR2.3-tg mice. Values are expressed as mean ± SEM. \*\*,  $P < 0.01$  (vs. gender-appropriate wild-type control).

*Altered bone morphology with reduced cortical area with no periosteal expansion in AR2.3-transgenic mice.* Morphological changes in calvaria from AR2.3-transgenic 8-week-old animals were characterized by H&E staining in fixed, decalcified and paraffin embedded calvarial sections from both sexes (in family 219; Fig. 5A). There was no difference between wild-type and AR2.3-transgenic animals in either gender, nor between AR2.3-transgenic independent families 219 and 223 (data not shown). In contrast, it is noteworthy that the calvaria from AR3.6-transgenic mice with periosteal overexpression demonstrate substantial calvarial thickening. We also evaluated AR protein expression *in vivo* by immunocytochemical analysis. Non-specific binding was blocked with normal goat serum, then slides were incubated with AR antibody. Immune complexes were detected after DAB staining, and slides were counterstained with hematoxylin. AR is brown and the nucleus is bluish purple after counterstaining;

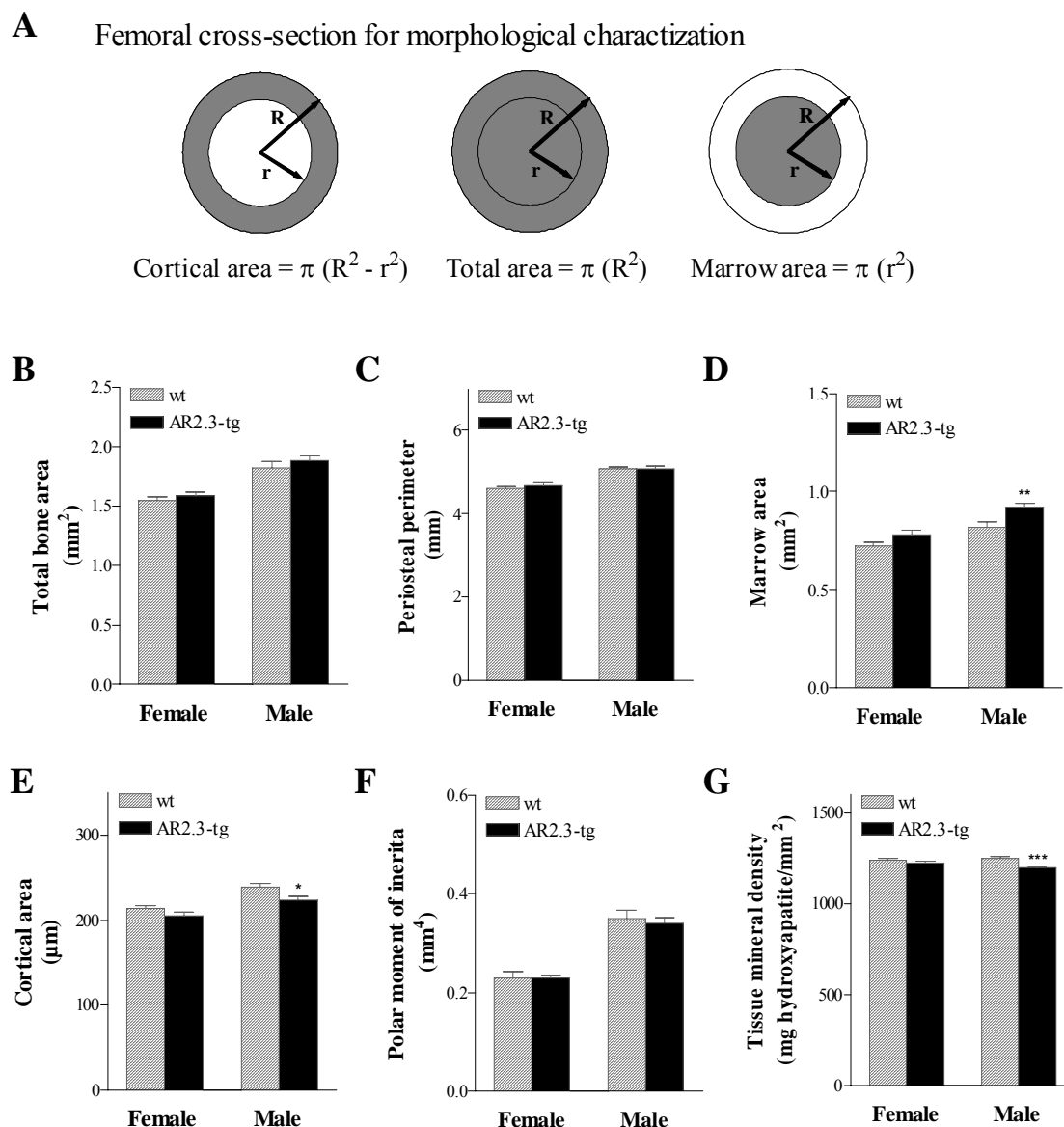
immunostaining represents both endogenous AR and the product of the AR2.3-transgene. The majority of osteoblasts and osteocytes demonstrated AR immunoreactivity. Both male and female AR2.3-transgenic



**Figure 4. DXA analysis of bone mineralization and body composition.** DXA was performed in 6 month old AR2.3-transgenic and littermate control mice. **A.** Areal bone mineral density (BMD) minus head was not adjusted for skeletal size. **B.** Bone mineral content (BMC). A trend for decreased BMC was noted in male AR2.3-tg mice (p=0.0571). **C.** Lean mass adjusted for total tissue mass. **D.** Fat mass adjusted for total tissue mass. Values are expressed as mean  $\pm$  SEM. n = 4-10.



**Figure 5. Histochemical and immunohistochemical analysis of calvaria from AR2.3-transgenic mice.** Calvaria were isolated from 8-week-old male and female mice from both AR2.3-transgenic (AR2.3-tg) lines (219 and 223) and wild-type (wt) littermate controls, and 5  $\mu$ m sections were subjected to either H&E staining after demineralization and paraffin embedding. Representative sections are shown. **A.** Calvaria from both 219 and 223 families were evaluated, and neither exhibited an increase in calvarial thickness (here shown 219 males vs. AR3.6-transgenic males). **B.** AR was detected with rabbit polyclonal antisera after DAB incubation. AR is brown and the nucleus is purple after counterstaining with hematoxylin. The majority of osteoblasts and osteocytes demonstrated AR immunoreactivity. As shown, there were no observable differences in overall AR expression between male and female animals (X40).

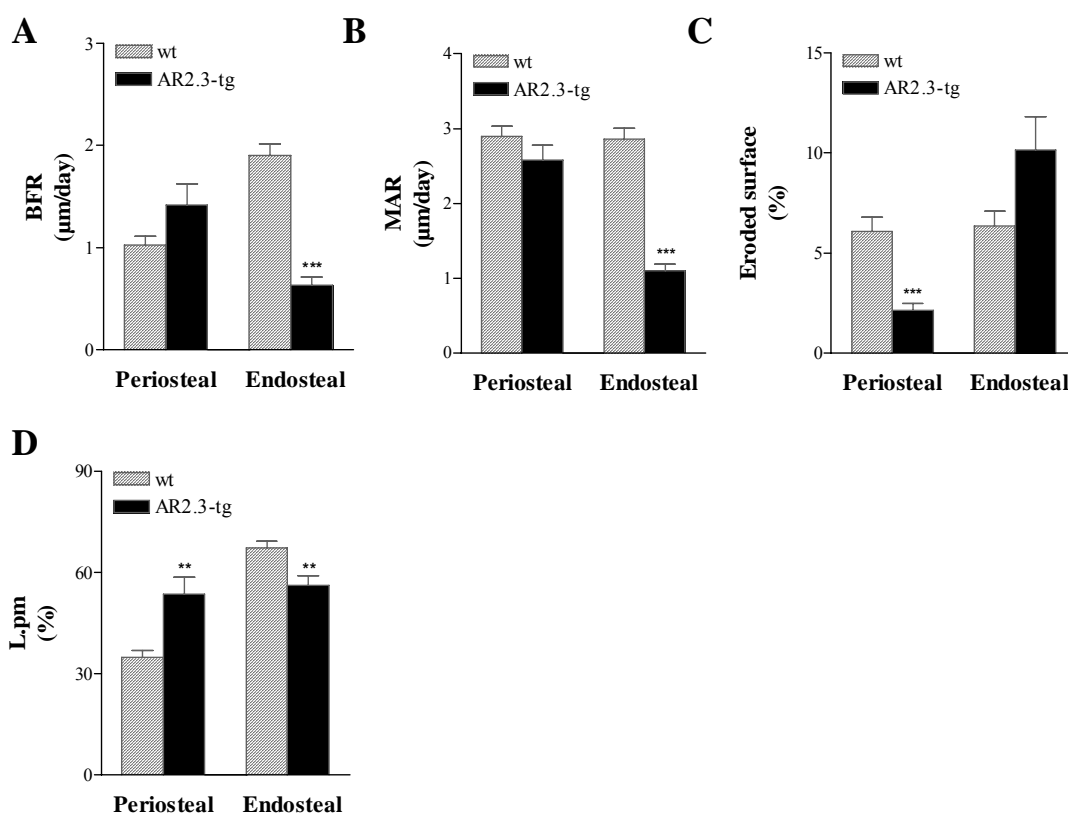


**Figure 6. Analysis of cortical morphology by  $\mu\text{CT}$  in AR2.3-transgenic mice.** Femurs were isolated from 8-week-old male and female wild-type (wt) or AR2.3-transgenic mice (AR2.3-tg) and subjected to high resolution  $\mu\text{CT}$  imaging at mid-diaphysis. **A.** Parameters for morphological characterization. **B.** Total bone area. **C.** Periosteal perimeter. **D.** Marrow cavity area. **E.** Cortical area. **F.** Polar moment of inertia. **G.** Tissue mineral density (TMD). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (all vs. gender-appropriate wt controls).

animals revealed higher level of AR expression as expected, with no notable difference between the sexes (Fig. 5B).

To better characterize the morphology of bone in AR2.3-transgenic mice, we examined femoral structure by high resolution  $\mu\text{CT}$  analysis. Quantification of the  $\mu\text{CT}$  analysis (described in Fig. 6A) demonstrated no effect of AR2.3-transgene expression on total bone area or surface periosteal perimeter (Fig. 6B, C). However, marrow cavity area was significantly increased (*i.e.*,

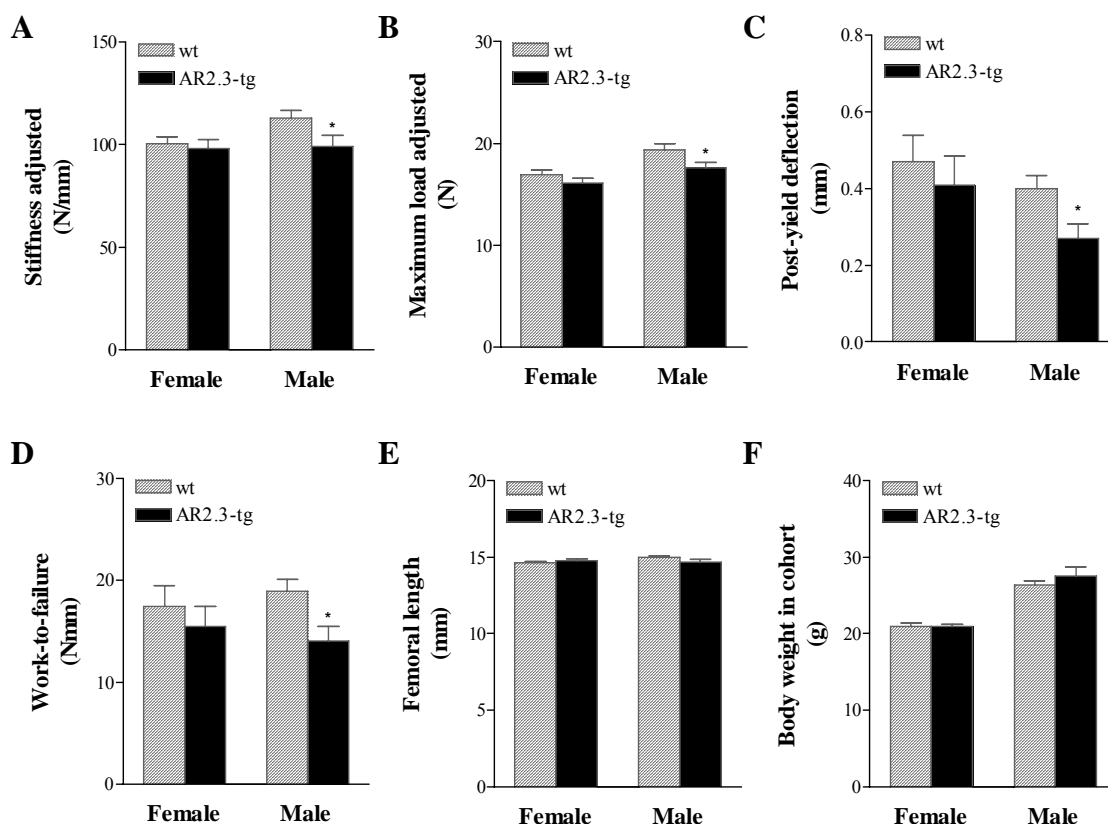
reduced infilling or increased resorption; Fig. 6D). Given no compensatory changes in the periosteal layer, this inhibition results in a significant reduction in cortical area (Fig. 6E) in male AR2.3-transgenic mice. These results demonstrate that AR overexpression in mature osteoblasts has substantial inhibitory effects on overall femoral cortical area in male mice. This morphological difference at the diaphysis was not observed in female AR2.3-transgenic mice, consistent with other observations that indicate that the female phenotype is mild at best. Based on the modest bone phenotype in the females, the changes noted in male AR2.3-transgenic mice are likely driven by AR transactivation associated with increased testosterone



**Figure 7. Rates of bone formation in AR2.3-transgenic mice at 8 weeks.** Dynamic histomorphometric analysis was performed in cortical bone by fluorescent imaging microscopy in AR2.3-tg males after double-label administration. 8-week-old male AR2.3-transgenic mice were pulsed with oxytetracycline followed 7 days later with calcein to fluorescently label mineralizing surfaces. Femurs were isolated and sectioned (100 µm) at the mid-diaphysis. Sections were subjected to fluorescent imaging microscopy to determine patterns of bone formation. **A.** Bone formation rate (BFR), **B.** mineral apposition rate (MAR), **C.** percent eroded surface and **D.** percent labeled perimeter (L.p.m) were determined at both the endosteal and periosteal surfaces in wild-type (wt) and AR2.3-transgenic (AR2.3-tg) mice. Data is mean ± SEM. n = 6-9 females, 3-7 males. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (vs. wt controls).

levels that occur as the males fully enter puberty (5-8 weeks). In addition, we also evaluated polar moment of inertia and tissue mineral density (TMD) at the mid-diaphysis. As shown in Fig. 6F, there was no significant effect on polar moment of inertia in either male or female AR2.3-transgenic mice, but males show a reduction in TMD ( $P < 0.001$ ; Fig. 6G), consistent with the reduction in BMC (Fig. 4B).

**Reduced endosteal bone formation in AR2.3-transgenic mice.** Because of the changes in cortical bone observed in the  $\mu$ CT analysis, fluorescent imaging was carried out at the femoral diaphysis. Fluorochromes were administered in 2-month old mice by double-label injection, with oxytetracycline followed by calcein to label deposition at the mineralizing front, and are representative of mineralization patterns only for the period of time that the labels are present.

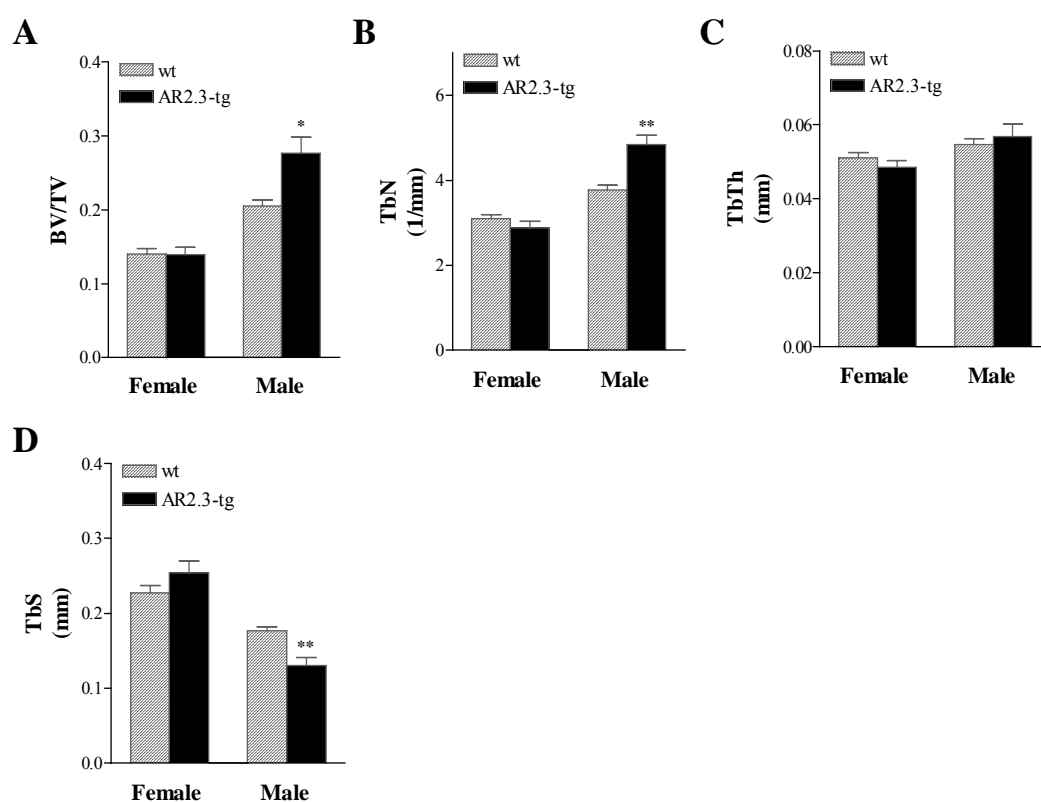


**Figure 8. Biomechanical analyses of bone quality in AR2.3-transgenic mice.** Femurs from wild-type (wt) and AR2.3-transgenic (AR2.3-tg) mice were isolated from 8-week-old mice to determine whole bone failure properties. Femurs were loaded to failure in 4-point bending and the stiffness and maximum load were calculated from the load-deflection curves and adjusted for body weight differences. **A.** Adjusted Stiffness. **B.** Adjusted Maximum Load. **C.** Post-yield deflection. **D.** Work-to-failure. **E.** Femoral Length. **F.** Body weight in cohort. The whole bone biomechanical properties are shown as mean  $\pm$  SEM,  $n = 7-14$ . Differences between genotypes were determined by Student's  $t$ -test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$  (vs. gender-appropriate wt controls).

Fig. 7 shows analysis of bone formation in images of fluorochrome labeling from femoral cross sections. The AR2.3-transgenic males (right panel) demonstrate a dramatic lack of labeling at the endosteal surface compared with wild-type controls (left panel). Consistent with these fluorescent images, dynamic histomorphometric analysis revealed inhibitory responses at the endosteal surface. Bone formation rate (BFR) at the endosteum was dramatically inhibited in AR2.3-transgenic mice ( $P < 0.001$ ; Fig. 7A). In addition, mineral apposition rate (MAR) was also inhibited at the endosteal surface ( $P < 0.001$ ; Fig. 7B). Analysis of labeled perimeter (L.pm) showed inhibition at the endosteal surface ( $P < 0.01$ ; Fig. 7D) but stimulation at the

periosteal surface ( $P < 0.01$ ). Eroded surface was reduced on the periosteum ( $P < 0.001$ ; Fig. 7C).

**Bone strength in male AR2.3-transgenic mice is reduced.** To analyze bone quality, whole bone failure properties were determined by loading femurs to failure in 4-point bending at 0.05 mm/s. Analysis shown in Fig. 8 revealed significant differences between wild-type and male AR2.3-transgenic animals (family 219) in whole bone biomechanical properties at 8 weeks. Male AR2.3-transgenic mice showed a significant decrease in stiffness ( $P < 0.05$ , Fig. 8A), with no effect noted in females. A modest but also significant decline in males was also noted in maximum load ( $P < 0.05$ , Fig. 8B). In addition, more robust inhibition was noted in post-yield deflection ( $P < 0.05$ , Fig. 8C) and work-to-failure ( $P < 0.05$ , Fig. 8D). Male AR2.3-transgenic mice in this cohort showed no difference in femoral length or weight (Fig. 8, E and F). Similar results were obtained with AR2.3-transgenic family 223.

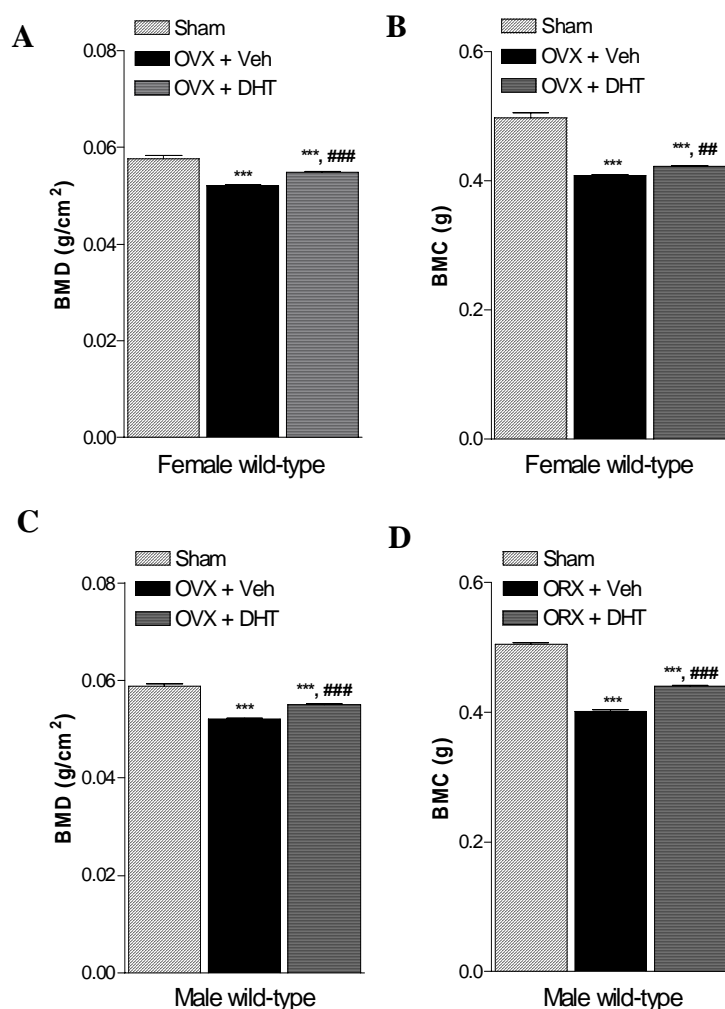


**Figure 9. Analysis of trabecular morphology by  $\mu$ CT in AR2.3-transgenic mice.** Femurs were isolated from 8-week-old male and female wild-type (wt) or AR2.3-transgenic mice (AR2.3-tg). Reconstructed images were evaluated for trabecular morphology in the distal metaphysis.  $\mu$ CT imaging and computer-aided analysis was used to derive measures of trabecular bone architecture and structure in the femoral metaphysis. Measurements included trabecular bone volume as a percent of tissue volume (BV/TV); trabecular number, spacing, and thickness (TbN, TbSp, TbTh). **A.** BV/TV. **B.** TbN. **C.** TbTh. **D.** TbSp. Values are expressed as mean  $\pm$  SEM. Unpaired, two-tailed  $P$  values were by Student's  $t$ -test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (vs. wt controls).

**Increased trabecular bone volume in AR2.3-transgenic mice.** Examination of  $\mu$ CT images suggested increased trabecular bone volume in male AR2.3-transgenic mice (Fig. 9). In order to examine the mechanism underlying the altered trabecular morphology, the metaphyseal

trabecular region in male AR2.3-transgenic mice was analyzed by static histomorphometry to characterize trabecular microanatomy and architecture (Fig. 9A-D). Male AR2.3-transgenic mice showed a 40% increase in trabecular bone volume as a percent of tissue volume (BV/TV;  $P < 0.05$ ), consistent with the  $\mu$ CT and histological analysis (not shown). The increase in trabecular bone volume was associated with a 26% increase in trabecular number (TbN;  $P < 0.01$ ), a 27% decrease in spacing (TbSp;  $P < 0.01$ ) but no effect on trabecular thickness (TbTh), consistent with a response to androgen.

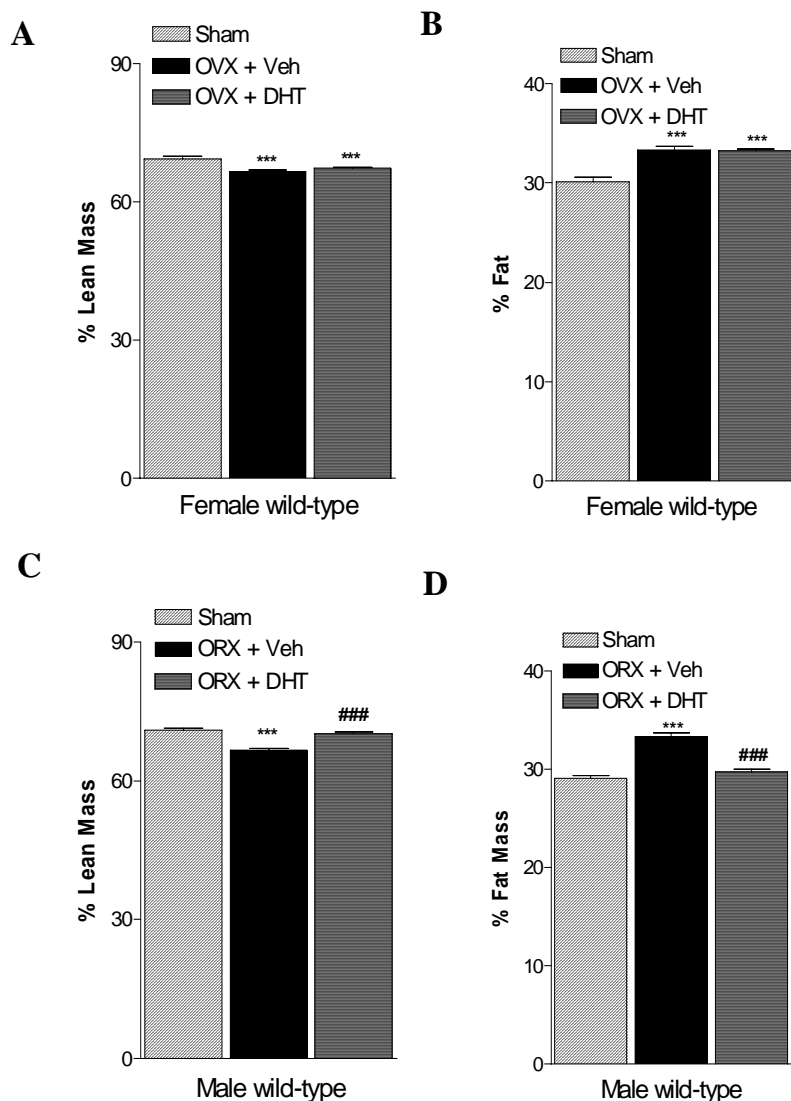
Final aspects of analysis include characterization of gene expression differences in RNA isolated from long bone (tibia) and from calvaria. These studies are nearly completed.



**Figure 10. Analysis of bone mineral after OVX/ORX in wild-type littermate control B6D2F1 mice.** Mice were ovariectomized (OVX) or orchidectomized (ORX) at 3 months of age. At 5 months, placebo or DHT pellets were implanted. After 6 weeks, bone mineral was characterized by DXA. **A.** Female BMD. **B.** Female BMC. **C.** Male BMD. **D.** Male BMC. Values are expressed as mean  $\pm$  SEM.  $n=10-24$  for females;  $n=14-20$  for males. One-way ANOVA followed by Tukey's post-hoc analysis was used to determine significance. \*\*\*,  $P < 0.001$  (vs. wt controls); ##,  $P < 0.01$  (vs. OVX placebo); ###,  $P < 0.001$  (vs. OVX or ORX placebo).

**Section 2:** With the loss of gonadal steroids (during menopause, andropause or surgical castration), bone turnover is increased dramatically (including increased bone resorption) such that bone strength is reduced. This high turnover state does not persist however, and after ~1 year in humans (or ~2 months in mice), turnover rates nearly return to baseline. We have proposed studies to examine two aspects of the phenotype we have observed in developing AR3.6-transgenic mice: inhibition of bone resorption on trabecular surfaces and mild stimulation at periosteal surfaces and in calvaria, reflecting the known physiological effects/roles of androgens. The hypothesis tested is that DHT transactivation of AR will protect against bone loss following hypogonadism following gonadectomy [either ovariectomy (OVX) or orchidectomy (ORX)] in both female and male mice. Two studies will separately test for anti-resorptive and anabolic actions of androgen replacement in the adult in both AR2.3- and AR3.6-transgenic mice.

Anabolic (increased bone formation) effects were examined in male and female mice castrated at ~3 months (initially in wild-type littermate controls) with steroid pellet replacement delayed for 2 months to allow turnover to stabilize. DHT was delivered for ~6 weeks, and mice were then evaluated for changes in bone mineral by dual energy x-ray absorptiometry (DXA) using a mouse PIXImus2 densitometer.



**Figure 11. Analysis of body composition after OVX/ORX in wild-type littermate control B6D2F1 mice.** Mice were ovariectomized (OVX) or orchidectomized (ORX) at 3 months of age. At 5 months, placebo or DHT pellets were implanted. After 6 weeks, body composition was characterized by DXA. **A.** Female % lean mass. **B.** Female % fat mass. **C.** Male % lean mass. **D.** Male % fat mass. Values are expressed as mean  $\pm$  SEM.  $n=10-24$  for females;  $n=14-20$  for males. One-way ANOVA followed by Tukey's post-hoc analysis was used to determine significance. \*\*\*,  $P < 0.001$  (vs. wt controls), ###,  $P < 0.001$  (vs. OVX or ORX placebo).

Analysis in wild-type mice (Fig. 10) shows the response to DHT after bone turnover has stabilized. As can be seen, in this setting androgen is dramatically anabolic in bone in the control mice. Both BMD and BMC are significantly increased in both male and female mice ( $P < 0.001$ ). As there has generally been difficulty establishing an anabolic response in mouse models, these results suggest that this model of stabilized turnover may reveal a stronger anabolic response in bone. Analysis in the AR2.3- and AR3.6-transgenic males and females is ongoing.

**Section 3: Androgens** have well characterized anabolic effects on muscle mass and strength. In the characterization of bone mineral in AR3.6-transgenic mice, we noted that male AR3.6-transgenic mice demonstrate a body composition phenotype, with decreased fat mass but increased lean mass. We have proposed the hypothesis that this change in body composition is a consequence of AR expression in stromal precursors; pluripotential cells with the ability to form a variety of tissues including muscle, fat, bone, cartilage

as outlined in the schema above. We have shown AR3.6-transgene expression in bone marrow stromal cells. With AR transactivation in male mice during development, we propose that bone marrow stromal cells respond by alteration of lineage commitment, away from the adipocyte and instead toward the myoblast to form more muscle in the AR3.6-transgenic mice. DXA analysis has been employed to characterize body compositional changes that occur in the adult setting after gonadectomy and DHT replacement (again in two settings; after a 2 month delay and immediately after OVX or ORX surgery in ongoing analyses).

Anabolic (increased lean mass) effects were examined in male and female mice castrated at 3 months (initially in wild-type littermate controls) with steroid pellet replacement delayed for 2 months to allow turnover to stabilize. DHT was delivered for 6 weeks, and mice were then evaluated for changes in body composition by DXA.

Analysis in wild-type mice (Fig. 11) shows the response to DHT after a sustained hypogonadal period. As expected, OVX and ORX produced a decrease in lean but an increase in fat mass in both males and females. In addition, in this hypogonadal setting androgen is dramatically anabolic to increase lean mass and reduce fat mass, but *only* in males. Interestingly, OVX females did not demonstrate a significant difference in body composition. These results suggest that females may not be as responsive to androgen treatment as an anabolic strategy to increase lean mass. Analysis in the AR2.3- and AR3.6-transgenic males and females is ongoing in both high and low turnover studies.

Finally, as a consequence of our studies on androgen action in osteoblasts and in particular work characterizing androgen signaling in the skeleton employing AR3.6-transgenic mice that resulted in publication of a review on androgen action (12), we were asked to participate in studies examining the effects of oxandrolone, an anabolic steroid given to severely burned patients, on osteoblast expression (please refer to Appendix 2).

Thus, primary outcomes of analyses through year two demonstrate that androgen action in the mature mineralizing osteoblast results in reduced bone biomechanics and matrix quality, with envelope-specific effects on bone formation centered on periosteal expansion. Compared with AR3.6-transgene overexpression throughout the osteoblast lineage (including bone marrow stromal cells, throughout osteoblast differentiation including osteocytes), AR2.3-transgenic males do not show reduction in femur length and demonstrate much less inhibition of whole bone strength properties including changes in stiffness, maximum load and work. However, AR2.3-transgenic mice show similar changes indicative of low turnover, and similar increases in brittleness (decreased post-yield deflection), suggesting an analogous change in matrix quality and/or mineralization in both AR3.6-transgenic and AR2.3-transgenic mice. In addition, as we have now published, androgen enhances osteoblast apoptosis (13). Finally, body composition is altered in AR3.6-transgenic mice, likely mediated by AR transgene expression in stromal precursors and alterations in lineage commitment. After a sustained hypogonadal period, androgen replacement dramatically improves BMD and BMC in both male and female control mice. However, the increase in fat and decrease in lean mass that is the consequence of OVX and ORX is not reversed in females, but is effectively treated in males by DHT replacement.

### Key Research Accomplishments

Accomplishments for 2005-2006 directly characterizing androgen action in the skeleton:

Peer reviewed publications:

- **Wiren KM**, Toombs AR, Semirale AA, Zhang X-W: Apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio. *Bone* 38:637–651, 2006

**\*Selected for journal cover**

- Bi LX, **Wiren KM**, Zhang X-W, Oliveira GV, Klein GL, Mainous EG, Herndon DN: Direct effect of oxandrolone treatment on human osteoblastic cells is modest. *J Burns Wounds in review*, 2006
- **Wiren KM**, Semirale A, Zhang X-W, Woo A, Tommasini SM, Price C, Schaffler M, Jepsen KJ: Dissection of androgen receptor signaling: reconsideration of direct anabolic action in mature bone. *J Clin Invest manuscript in preparation*, 2006.

Abstracts:

- **Wiren KM**: Direct androgen action in the skeleton. (Wellness/Fitness Research Abstract #121) PRMRP Military Health Research Forum, 2006
- Semirale A, Zhang X-W, **Wiren KM**: Androgen receptor overexpression in precursor cells and not mature osteoblasts influences body composition by reducing adiposity and increasing lean mass. (Abstract#SU466 ) *J Bone Miner Res*, 2006
- **Wiren KM**, Zhang X-W, Semirale A, Price C, Woo A, Jepsen KJ: Targeted androgen action in mature vs. preosteoblasts eliminates periosteal stimulation and reduces bone quality during growth. (Abstract#1015 ) *J Bone Miner Res*, 2006

**\*Selected for Oral Presentation ASBMR**

**Reportable Outcomes**

One peer-reviewed publication (with one more submitted and a second in preparation) and three abstracts were published in 2005-2006 characterizing androgen action in the skeleton. In the work summarized here, we have also successfully characterized a second distinct AR transgenic family set with skeletally-targeted AR overexpression at 2 months of age. In contrast to the original AR3.6-transgenic family, the AR2.3-transgenic family demonstrates overexpression that is limited to mature osteoblasts and mineralizing osteocytes. Finally, the data generated by this grant provided evidence that AR signaling also influences developmental processes during skeletal modeling. This data helped secure an R01 grant from the NIH (DK067541) entitled, "Androgen action in bone: overexpression of AR". The goals of this R01 grant are to characterize the consequence of androgen signaling during early development.

**Conclusions**

These results have provided new insight into the importance of androgen action, through distinct AR transactivation, in mediating bone quality and changes that underlie envelope-specific responses. Our investigations of the mouse skeleton reveal that androgen signaling in immature osteoblasts and periosteal cells increases bone formation at the periosteal envelope (particularly in calvaria) and also influences processes that determine body composition and whole bone strength. In contrast, analysis presented here focusing on AR2.3-transgenic mice suggests that signaling in mature osteoblasts/osteocytes primarily mediates the effects of androgens on matrix quality and/or mineralization (with a negative impact), and at least partially influences the effects of androgens to reduce bone turnover. These data suggest that a similar relationship may also exist in the human skeleton. In sum, these results indicate that mature osteoblasts are important mediators of androgen action to influence matrix quality and influence turnover parameters, but immature osteoblast lineage and periosteal cells are the major contributors to envelope-specific alterations in bone formation and stromal lineage commitment can be influenced by AR activation. These results raise concerns that with increasing androgen action (as for example associated with anabolic steroid abuse) in young animals with still-growing skeletons, the bone matrix material is likely to be more stiff, less ductile and more damageable, and suggest that young anabolic steroid abusers may be at higher risk of stress fractures. However, our preliminary analyses in the mature adult indicate that androgen may be useful to treat reductions in bone mineral and furthermore, improve body composition in males.

These results support the central hypothesis of distinct androgen signaling throughout osteoblast differentiation. Thus androgen action in the skeleton is complex and will likely not provide for improved skeletal dynamics in the still-growing skeleton, although the effect in mature adults and in the hypogonadal state is currently being evaluated and suggest more positive outcomes. One of the side effects of enhanced androgen signaling is altered whole bone quality and susceptibility to damage in the young, which may be revealed under extreme physical conditioning such as that experienced during military training. However, it appears that androgen may be effective in improving body composition, including increased lean and reduced fat mass, but only in males.

## References

1. **Sambrook P, Seeman E, Phillips S, Ebeling P** 2002 Preventing osteoporosis: outcomes of the Australian Fracture Prevention Summit. *Med J Aust* 176:S1-16
2. **Smith M** 2002 Osteoporosis and other adverse body composition changes during androgen deprivation therapy for prostate cancer. *Cancer Metastasis Rev* 21:159-166
3. **Ross R, Small E** 2002 Osteoporosis in men treated with androgen deprivation therapy for prostate cancer. *J Urol* 167:1952-1956
4. **Ray N, Chan J, Thamer M, Melton Lr** 1997 Medical expenditures for the treatment of osteoporotic fractures in the United States in 1995: report from the National Osteoporosis Foundation. *J Bone Miner Res* 12:24-35
5. **Riggs B, Melton Lr** 1995 The worldwide problem of osteoporosis: insights afforded by epidemiology. *Bone* 17(5 Suppl):505S-511S
6. **Riggs B, Khosla S, Melton III L** 2002 Sex steroids and the construction and conservation of the adult skeleton. *Endo Revs* 23:279-302
7. **Rako S** 2000 Testosterone supplemental therapy after hysterectomy with or without concomitant oophorectomy: estrogen alone is not enough. *J Womens Health Gend Based Med* 9:917-923
8. **Hashimoto J, Beadles-Bohling A, Wiren K** 2004 Comparison of RiboGreen and 18S rRNA quantitation for normalizing real-time RT-PCR expression analysis. *Biotechniques* 36:54-60
9. **Kalajzic Z, Liu P, Kalajzic I, Du Z, Braut A, Mina M, Canalis E, Rowe D** 2002 Directing the expression of a green fluorescent protein transgene in differentiated osteoblasts: comparison between rat type I collagen and rat osteocalcin promoters. *Bone* 31:654-660
10. **Wiren K, Zhang X-W, Toombs A, Gentile M, Kasparcova V, Harada S-I, Jepsen K** 2004 Targeted overexpression of androgen receptor in osteoblasts: unexpected complex bone phenotype in growing animals. *Endocrinology* 145:3507-3522
11. **Roodman G** 2006 Regulation of osteoclast differentiation. *Ann N Y Acad Sci* 1068:100-109
12. **Wiren K** 2005 Androgens and bone growth: its location, location, location. *Curr Opin Pharmacol* 5:626-632
13. **Wiren K, Toombs A, Semirale A, Zhang X-W** 2006 Apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio. *Bone* 38:637-651

Appendix 1 follows:

Wiren KM, Toombs AR, Semirale AA, Zhang X-W: Apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio. Bone 38:637–651, 2006

\*Selected for journal cover

# Osteoblast and osteocyte apoptosis associated with androgen action in bone: Requirement of increased Bax/Bcl-2 ratio<sup>☆</sup>

Kristine M. Wiren<sup>a,b,\*</sup>, Amber R. Toombs<sup>a</sup>, Anthony A. Semirale<sup>a,b</sup>, Xiaowei Zhang<sup>a,b</sup>

<sup>a</sup> Veterans Affairs Medical Center, Portland, OR 97239, USA

<sup>b</sup> Departments of Medicine and Behavioral Neuroscience, Oregon Health and Science University, Portland, OR 97239, USA

Received 19 July 2005; revised 7 October 2005; accepted 14 October 2005

Available online 18 January 2006

## Abstract

Both the number and the activity of osteoblasts are critical for normal bone growth and maintenance. Although a potential role for estrogen in protection of bone mass through inhibition of osteoblast apoptosis has been proposed, a function for androgen is much less clear. The aim of this study was to establish a direct role for androgen to influence osteoblast apoptosis both in vitro and in vivo. AR-MC3T3-E1 cells, with androgen receptor (AR) overexpression controlled by the type I collagen promoter, were treated with the non-aromatizable androgen 5 $\alpha$ -dihydrotestosterone (DHT). Apoptosis was assessed by three different techniques including DNA fragmentation, caspase-3 activation, and changes in mitochondrial membrane potential. Transactivation of AR by DHT enhanced apoptosis while 17 $\beta$ -estradiol (E<sub>2</sub>) treatment reduced apoptosis in both proliferating preosteoblasts and mature osteocyte-like cells. To explore mechanism, the apoptosis regulators Bcl-2 (antiapoptotic) and Bax (proapoptotic) were evaluated. Western analysis revealed that DHT decreased Bcl-2 resulting in a significantly increased Bax/Bcl-2 ratio. Regulation of Bcl-2 was post-transcriptional since *bcl-2* mRNA levels were unaffected by DHT treatment. Furthermore, ubiquitination of Bcl-2 was increased and serine phosphorylation was reduced, consistent with inhibition of MAP kinase signaling by DHT. Increased Bax/Bcl-2 ratio was essential since either Bcl-2 overexpression or Bax downregulation by RNA interference (RNAi) partially abrogated or reversed DHT-enhanced osteoblastic apoptosis. In order to establish physiologic significance in vivo, AR-transgenic mice with AR overexpression in the osteoblast lineage and thus enhanced androgen sensitivity were characterized. In male AR-transgenic mice, increased osteoblast apoptosis was observed in vivo even in association with new bone formation. Thus, although estrogen can be antiapoptotic, androgen stimulates osteoblast and osteocyte apoptosis through an increased Bax/Bcl-2 ratio even in anabolic settings. These results identify a new mechanism for androgen regulation of osteoblast activity distinct from estrogen, and suggest that enhanced apoptosis can be associated with anabolic stimulation of new bone growth. Androgens thus play a distinct role in skeletal homeostasis.

© 2005 Elsevier Inc. All rights reserved.

**Keywords:** Osteoblast; Apoptosis; Androgen receptor; Androgen; Estrogen

## Introduction

The skeleton is an important target organ for androgen action, distinct from estrogenic effects. Androgen receptors (AR) are expressed in osteoblasts, the cells responsible for osteogenesis. AR levels are increased in osteoblasts after

androgen exposure [56,58] and as cells differentiate into a mature osteocytic phenotype [55]. The importance of AR signaling to influence skeletal homeostasis has been clearly demonstrated in both human [32] and mouse [51] models with androgen insensitivity, and in global AR null models [42,62]. Characterization of AR-transgenic mice with skeletally targeted AR overexpression has demonstrated proof of principle that direct androgen signaling in the osteoblast is an important mediator of androgen action in bone [59]. While the association of androgen with skeletal health and normal bone homeostasis is well established (for review, see [52]), the physiologic responses and molecular pathways influenced by androgen in

<sup>☆</sup> Presented in part at the 26th Annual Meeting of the American Society for Bone and Mineral Research, Seattle, Washington, October 1–5, 2004.

\* Corresponding author. Department of Medicine, Oregon Health and Science University, VA Medical Center, Research Service P3-R and D39, 3181 SW US Veterans Hospital Road, Portland, OR 97239-2964, USA. Fax: +1 503 2735351.

E-mail address: [wirenk@ohsu.edu](mailto:wirenk@ohsu.edu) (K.M. Wiren).

bone remain poorly characterized. The influence of estrogen on bone cell life span is an area of active investigation since it may be important in maintenance of bone mass, and play a role in the development of osteoporosis [48]. However, the effect of androgen is not well understood and is complicated by the fact that testosterone is the substrate for estradiol synthesis through aromatase activity. Thus, a controversy exists surrounding androgen's specific role(s) in bone homeostasis.

Androgen has been shown to influence bone cells in a complex fashion. For example, the effect of androgen on osteoblast proliferation has been shown to be biphasic in nature, with enhancement following short or transient treatment but significant inhibition following longer treatment (see [57]). Continuous treatment with the non-aromatizable androgen 5 $\alpha$ -dihydrotestosterone (DHT) in proliferating preosteoblasts also resulted in decreased cell viability [57]. Reduced viability was associated with overall reduction in mitogen-activated (MAP) kinase signaling and with inhibition of *elk-1* gene expression, protein abundance, and extent of phosphorylation. Importantly, inhibition of MAP kinase signaling was observed both in normal primary rat calvarial cultures and in a model of enhanced androgen responsiveness, AR-MC3T3 cells. This result contrasts with stimulation of MAP kinase signaling and AP-1 transactivation observed with brief androgen exposure, that may be mediated through non-genomic mechanisms [24,28,63]. Since enhanced apoptosis is frequently associated with inhibition of MAP kinase signaling, the aim of the present study was to define the effects of androgen administration on osteoblast apoptosis in vitro and in vivo.

Apoptosis, or programmed cell death, is an essential process that is important for maintaining tissue homeostasis under normal conditions, in response to environmental insults and plays a vital role during embryogenesis that includes shaping of developing tissues [1]. In bone, apoptosis is important during embryonic limb development, skeletal maturation and modeling, adult bone turnover by remodeling, and during fracturing healing and regeneration [19]. One of the best characterized mechanisms to induce cell death is the release of proapoptotic proteins from the mitochondria into the cytosol (for a recent review, see [4]). Members of the Bcl-2 family, including Bcl-2 and Bax, are central regulators of apoptosis that promote (Bax) or inhibit (Bcl-2) cell death. Although a competition between Bax and Bcl-2 exists, each is able to regulate apoptosis independently [26]; thus, the ratio of Bax/Bcl-2 is an important indicator of apoptosis.

Previous reports indicate that Bcl-2 can be phosphorylated, and that phosphorylation of Bcl-2 is closely associated with regulation of apoptosis [8,14]. For example, positive regulation of Bcl-2 levels through MAP-kinase-mediated phosphorylation has been demonstrated, with activation of p44/42 MAP kinase inhibiting both downregulation of Bcl-2 protein and subsequent apoptosis [41]. The converse has also been observed, with inhibition of MAP kinase signaling shown to reduce Bcl-2 phosphorylation at consensus MAP kinase sites, leading to enhanced ubiquitination and ensuing Bcl-2 degradation in proteasomes [5,9]. Androgen treatment has been reported to reduce Bcl-2 levels [6,20] and enhance apoptosis [54,64] in

some tissues. Although we have demonstrated androgen-mediated reductions in MAP kinase signaling with chronic treatment [57], the impact of androgen treatment on levels of antiapoptotic Bcl-2 in osteoblasts has not been previously characterized.

Most analyses regarding effects of sex steroids on osteoblast apoptosis indicate that estrogen may be important in inhibiting apoptosis to increase osteoblast life span [39]. Few studies have directly analyzed the specific effects of androgens, however, leading to controversy regarding the nature of androgen signaling. In addition, it has been postulated that AR and estrogen receptor (ER) can activate an antiapoptotic non-genomic signaling pathway in osteoblasts with the same effectiveness regardless of whether the actual steroid ligand is an androgen or an estrogen [27]. Recent data, however, suggests that distinct, genomic signaling may be the more significant regulator in vivo [36,44,50]. In this study, the specific effect of androgen on osteoblast apoptosis through AR transactivation was examined in vitro with AR-MC3T3 cultures and in vivo in AR-transgenic mice. Interestingly, one facet of the low turnover bone phenotype observed in young male AR-transgenic mice is an anabolic response to androgen signaling [59], seen with an enhanced bone formation rate at the periosteum of long bones and with calvarial thickening. The effect of androgen on osteoblast apoptosis in an anabolic setting has not previously been examined. To gain further insights into the specific effect of androgen vs. estrogen on osteoblast apoptosis, we employed two novel models of enhanced androgen responsiveness; clonal col3.6 AR-MC3T3 cells (hereafter referred to as AR-MC3T3) stably transfected with full-length AR under control of 3.6 kb of the type I collagen promoter, and male col3.6 AR-transgenic mice (hereafter referred to as AR-transgenic mice). Results indicate dichotomous regulation of osteoblast apoptosis by androgen vs. estrogen, with chronic administration of androgen stimulating but estrogen inhibiting apoptosis.

## Materials and methods

### Reagents

All the media, buffers, supplements, and reagents for cell culture were obtained from GIBCO BRL-Life Technologies (Grand Island, NY) or Sigma Chemical Co. (St. Louis, MO). Steroid hormones and other reagents were obtained from Sigma Chemical Co. The active metabolite hydroxyflutamide ( $\alpha,\alpha,\alpha$ -trifluoro-2-methyl-49-nitro-*m*-lactotoluidide, SCH 16423) was kindly provided by Schering-Plough Corp. (Madison, NJ). Hydroxyflutamide, an androgen receptor antagonist, was added to the cultures 30 min before hormone addition.

### Cell culture

Cells stably transfected with AR (AR-MC3T3) or  $\beta$ -galactosidase ( $\beta$ gal-MC3T3) under the control of the rat 3.6 kb  $\alpha$ 1(I)-collagen promoter were created as previously described [57]. Cultures were maintained in minimal essential media (MEM) with 2.38 g/L HEPES and 2.2 g/L NaHCO<sub>3</sub> buffer, supplemented with 5% calf serum (CS). These cells were maintained throughout the study in the presence of G418 geneticin sulfate at 500  $\mu$ g/ml. Cultures were treated with steroids in 5% charcoal-stripped CS. For appropriate osteoblast differentiation, cultures were switched at confluence to media containing 50  $\mu$ g/ml ascorbic acid and 10 mM  $\beta$  glycerophosphate. Osteocytic cultures were

derived from cells grown 20–30 days (see [31,55,61]). Steroids were dissolved as stocks in ethanol and used at concentrations from  $10^{-12}$  M to  $10^{-8}$  M. The final ethanol concentration in the media was no higher than 0.1%.

### Transient transfection, luciferase reporter assay

The potential of  $E_2$  to stimulate ER transcriptional activation was determined using the  $E_2$  response element (ERE) basal promoter-luciferase (4ERE-TATA-luciferase; ERE-luc) reporter construct containing 4 EREs linked to a minimal promoter, kindly provided by Dr. David Shapiro. ERE-luc was used to confirm that overexpression of AR did not influence transactivation of other steroid hormone receptors. AR-MC3T3 cells were grown in 6-well plates for 24 h before transfection. AR-MC3T3 cells were transfected with FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN) using 1.5  $\mu$ g of ERE-luc and 0.5  $\mu$ g  $\beta$ gal expression vector for normalization in each well. Cultures were treated with vehicle or  $10^{-12}$ – $10^{-8}$  M  $E_2$ , with combined treatment with  $E_2$  and  $10^{-8}$  M DHT, or with DHT alone for 54 h. Cells were harvested and extracted in 300  $\mu$ l reporter lysis buffer (Promega Corp, Madison WI). Luciferase and  $\beta$ gal activities were analyzed as previously reported [57]. The data shown represent the mean  $\pm$  SEM from triplicate samples, performed in independent transfections two to three times.

### Western blot analysis and immunoprecipitation

Protein was extracted from proliferating cell cultures as previously reported [55]. Polyclonal rabbit antibody recognizing AR (PA1-111A, Affinity Bioreagents Inc.) was used at 4  $\mu$ g/ml. Both Bcl-2 and anti-mouse Bax polyclonal antibody ( $\Delta$ 21) specific for all 1–171 amino acids but the carboxy-terminal 21 amino acids (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were diluted at 1:200. As a loading control for total protein, Western blot analysis was performed with an anti- $\alpha$ -tubulin antibody at 1:1000 (Sigma, Saint Louis, MO). Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Bio-Rad Laboratories, Richmond, CA) at 1:2000. Bound antibodies were visualized by ECL (Amersham Pharmacia Biotech, Piscataway, NJ) on a Kodak X-AR5 autoradiographic film.

For the detection of phosphorylated or ubiquitinated forms of Bcl-2, AR-MC3T3 cells were treated with  $10^{-8}$  M of DHT for 72 h. To stimulate extracellular signal regulated kinase (ERK) activity, cultures were first starved in serum-free medium for 1 h with a subsequent addition of 5% charcoal stripped serum for 2 h. Equal amounts of protein were immunoprecipitated with anti-Bcl-2 antibody. Immunocomplexes were collected with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.), resolved by 12% SDS-PAGE, and transferred onto Immobilon-P PVDF membrane. Western blot analysis after immunoprecipitation was carried out with either antiphosphoserine-specific antibody at 0.1  $\mu$ g/ml (clone 4A9; Alexis Biochemicals, San Diego, CA), anti-Bcl-2 antibody as described above, or antiubiquitin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:300 dilution. Western blot analysis of extracts from AR-MC3T3 cells 48 h following mock transfection or transfection with the Bcl-2 expression plasmid or Bax siRNA was also performed. Quantitative analysis of the proteins was performed by volume densitometry using Optiquant Software (PerkinElmer Life and Analytical Sciences, Inc, Boston, MA) after scanning of the film (ScanMaker 9800 XL, Microtek, Carson, CA) in the linear range. Apoptosis data were expressed as the Bax/Bcl-2 ratio. Immunoprecipitation data are presented as the protein to  $\alpha$ -tubulin ratio to correct for variations in protein loading, and then normalized to control values for comparison between treatments. The data shown represent the mean  $\pm$  SEM.

### AR quantification

Both AR-MC3T3 and control  $\beta$ gal-MC3T3 stable cultures were grown for 5 to 25 days, and AR binding analysis was performed as previously described [57]. ARs were quantitated by titration analysis using [ $^3$ H]methyltrienolone ([ $^3$ H]R1881) (New England Nuclear, specific activity 70–87 Ci/mM). Specific [ $^3$ H]R1881 binding capacity was estimated by one site binding with non-linear regression and Scatchard plot analysis using Prism v4 software (GraphPad Software, Inc, San Diego, CA). The high affinity ( $K_d = 1.0$ – $5.0 \times 10^{-10}$  M), specific binding of [ $^3$ H]R1881 was expressed in fmol/mg DNA and sites/cell.

The sensitivity for the detection of specific androgen binding using these methods was 5 fmol/ml.

### Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and primer sequence

Confirmation of endogenous AR and colAR transcript expression was performed using qRT-PCR with the iCycler IQ Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) on DNase-treated total RNA as previously described [59]. AR transgene mRNA was amplified with 5'-GCATGAGCCGAAGCTAAC-3' and 5'-GAACGCTCCTCGATAGGTCTTG-3'. These primers were designed to specifically amplify the transgene using sites in the collagen untranslated region and part of the AR sequence. Endogenous mouse AR mRNA was amplified with 5'-GGAATTCGGTGAAGCTACA-3' and 5'-CCGGGAGGTGCTATGT-3'. To determine the mRNA levels of *bcl-2* and *bax* after treatment with DHT or  $E_2$ , murine *bcl-2* mRNA was amplified with 5'-TTCGCAGAGATGTCCAGTCA-3' and 5'-CACCCCATCCCTGAA-GAGTT-3'; murine *bax* mRNA was amplified with 5'-CCAAGAAGCTGAGC-GAGTGT-3' and 5'-TGTCCACGTCAGCAATCATC-3'. Relative expression of the RT-PCR product was determined using the comparative  $\Delta\Delta C_t$  method after normalizing expression with either 18S rRNA or fluorescence to the specific RNA binding dye RiboGreen (Molecular Probes, Eugene, OR, USA) as previously described [15]. Real-time qRT-PCR efficiency was determined for each primer set using a 5-fold dilution series of total RNA and did not differ significantly from 100%. Following PCR, specificity of the PCR reaction was confirmed with melt curve analysis. Amplicons were also sequenced for confirmation. Data are presented as mean  $\pm$  SEM.

### Assessments of apoptosis

AR-MC3T3 cells were plated in 6-well dishes and grown for either 5 days (proliferating) or 29 days (osteocyte-like) in the continuous presence of either vehicle or  $10^{-8}$  M DHT or  $10^{-8}$  M  $E_2$ . Apoptosis was induced either with 50  $\mu$ M etoposide (ETOP) for 18 h before harvest or by serum starvation for 48 h, replacing serum with 0.1% bovine serum albumin. Three independent methods were employed for analysis of apoptosis after these treatments.

### Cytoplasmic nucleosome enrichment

Quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in combined attached and detached cell pools of AR-MC3T3 treated as described above used the Cell death ELISA<sup>Plus</sup> (Roche Diagnostics, Indianapolis, IN) kit. Absorbance was measured at 405 nm, and data are expressed as the nucleosome enrichment factor =  $A_{\text{treated}}/A_{\text{untreated}}$ .

### Caspase-3/CPP-32 activity

Apoptosis-induced activation of caspase-3 activity in treated AR-MC3T3 cultures was assessed by measuring colorimetric substrate DEVD-pNA cleavage (BioVision, Inc.) according to manufacturer's suggestions in a homogenate containing 50  $\mu$ g protein.

### Mitochondrial membrane potential

Loss in mitochondrial membrane potential ( $\Delta\Psi_{\text{mito}}$ ) in treated AR-MC3T3 cells after induction of apoptosis was detected using ApopAlert Mitochondrial Membrane Sensor kit (BD Biosciences Clontech, Palo Alto, CA). AR-MC3T3 cultures were rinsed with serum-free media, stained with BD MitoSensor Dye at 37°C for 20 min, and analyzed by fluorescence microscopy.

### In vivo histological analysis

AR-transgenic mice with AR overexpression in the osteoblast lineage were employed for in vivo histological analysis. AR-transgenic mice, previously generated and characterized [59], were healthy and transmitted the transgene at the expected frequency. For experiments, hemizygous littermates were obtained by mating transgenic founders with B6D2F1 wild-type mice of both genders. Calvaria were isolated from AR-transgenic mice and littermate controls at 2

months and 6 months old, fixed in 4% paraformaldehyde, decalcified in Immucal (Decal Corp., Tallman, NY), and then processed for paraffin embedding as previously described [59]. 5–6  $\mu\text{m}$  sections were cut and stained with hematoxylin and eosin (H&E). New bone growth was localized in calvarial cross sections using van Geison staining to identify collagen synthesis that has formed within 4 days of isolation [17]. Apoptosis-induced DNA fragmentation in bone sections was assessed by means of Terminal-dUTP-Transferase-Nick-End Labeling (TUNEL) staining using TdT-FragEL DNA fragmentation detection kit (Oncogene research products, Boston, MA) following manufacturer's recommendations. Detection was performed using a streptavidin–horseradish peroxidase conjugate and 3,3'-diaminobenzidine as a color substrate. Sections were counterstained with methyl green, mounted, and viewed under a microscope. Apoptotic cells are identified with condensed nuclei and dyed brown. Positive and negative controls were always included. Positive control sections were treated with 1  $\mu\text{g}/\mu\text{l}$  DNase I in TBS with 1 mM  $\text{MgSO}_4$  for 20 min at room temperature following proteinase K treatment. Negative control sections were incubated without TdT.

#### Transient transfection, overexpression, and RNA interference (RNAi) analysis

Experiments were performed to alter Bax/Bcl-2 ratio by transient transfections. Bcl-2 overexpression was achieved using pCMV-Bcl-2 expression construct (kindly provided by Dr. Stanley Korsmeyer, Harvard Medical School). AR-MC3T3 cells were plated onto 6-well dish and transfected with 2  $\mu\text{g}$  of DNA per well using FuGENE 6 transfection (Roche, Basel, Switzerland) reagent at a 6:1 ratio (FuGene6:  $\mu\text{g}$  DNA). To inhibit *bax* gene expression, an oligonucleotide-based technique with double-stranded short interfering RNAs (siRNA) validated for *bax* was used, following manufacturer's instructions. Bax siRNA was introduced via transient transfection with SureSilencing Mouse Bax siRNA Kit (SuperArray Bioscience Corp., Frederick, MD) and Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA). Transfections included negative control siRNA populations. AR-MC3T3 cells were transfected with indicated sequences (pCMV-Bcl-2 or Bax siRNA). Cultures were exposed to  $10^{-8}$  M DHT for 30 h followed by 18 h-treatment with 50  $\mu\text{M}$  etoposide and then isolated at day 5. Apoptosis was evaluated using the Cell Death oligonucleosome assay in transfected and mock-transfected cultures. The data are expressed as the mean  $\pm$  SEM. Confirmation of alteration of protein levels of Bcl-2 and Bax was determined with Western blotting (see Fig. 6).

#### Statistical analysis

All data were analyzed using Prism v4.0 software (GraphPad Software, Inc.; San Diego, CA). Values from duplicate or triplicate wells from two to three experiments were used for statistical analysis with Student's *t* test or one-way ANOVA followed by post hoc analysis with Newman–Keuls Multiple Comparison Test. Differences of  $P < 0.05$  were considered statistically significant. Results are presented as the mean  $\pm$  SEM.

## Results

#### Model of enhanced androgen responsiveness

AR levels are low as osteoblasts proliferate but increase during differentiation in vitro to reach maximal levels in mature osteocytic-like cells [55]. In order to enhance androgen responsiveness, particularly in early proliferating osteoblasts, we have employed cultures of the immortalized mouse calvarial osteoblastic cell line MC3T3-E1 stably transfected with an AR expression construct under the control of 3.6 kb of the rat  $\alpha 1(\text{I})$ -collagen promoter (for details, see reference [57]). AR-MC3T3 cells were used as stable pools and grown under selection with G418.

To determine total AR levels throughout in vitro differentiation, both proliferating and osteocytic AR-MC3T3 cultures were characterized with AR binding and Scatchard plot analyses (Table 1). As can be seen, there is an approximately 3-fold elevation in AR binding between control  $\beta\text{gal}$ -MC3T3 vs. AR-MC3T3 cultures in proliferating cultures at day 5. In mature osteoblastic/osteocytic cultures at day 25, there is also approximately 3-fold elevation in AR levels compared to control cultures. In addition, AR levels nearly doubled during differentiation in AR-MC3T3 cultures, with a less robust increase in control cultures. Analysis by two-way ANOVA demonstrated that both cell line and time in culture significantly influenced AR levels. AR protein abundance was also determined by Western analysis in whole cell lysates isolated during proliferation (day 5) and at a more mature osteoblast stage (day 20). As shown in Fig. 1A, total AR levels (from both endogenous AR and colAR transgene expression) are elevated in mature cultures. We next analyzed expression of the colAR transgene by qRT-PCR analysis using total RNA isolated from AR-MC3T3 cultures with primers specific for the colAR transgene or for endogenous AR (Fig. 1B). AR-transgene expression was elevated approximately 1.7-fold relative to the endogenous AR gene.

In order to evaluate whether overexpression of AR in these cultures alters steroid receptor signaling in a generalized non-specific fashion, we characterized transactivation mediated by a distinct steroid receptor, i.e., estrogen transactivation of ERs in AR-MC3T3 cultures. ER activity was evaluated in transient transfections using the ERE reporter construct ERE-luc. Cells were treated for 54 h with  $\text{E}_2$  in a dose response ( $10^{-12}$  M– $10^{-8}$  M) in the presence or absence of  $10^{-8}$  M DHT. AR-MC3T3 cultures demonstrated a dose-dependent stimulation in ER transactivation with approximately 1.6-fold elevation with  $10^{-8}$  M  $\text{E}_2$ . Importantly,  $\text{E}_2$  signaling was not altered during coincident AR transactivation by cotreatment with combined  $10^{-8}$  M  $\text{E}_2$  and DHT, nor was DHT alone sufficient to activate ERE-dependent ER transactivation (Fig. 1C).

Table 1  
Analysis of AR levels with differentiation in AR-MC3T3 cultures

Tissue/cells	$K_d \times 10^{-10}$ M	Bmax (fmol/mg DNA)	Capacity (sites/cell)
d5 $\beta\text{gal}$ -MC3T3	$1.9 \pm 0.45$	$644 \pm 138$	$2323 \pm 496$
d5 AR-MC3T3	$2.2 \pm 0.05$	$1491 \pm 16$	$5383 \pm 60$
d15 $\beta\text{gal}$ -MC3T3	$1.5 \pm 0.03$	$991 \pm 160$	$3576 \pm 576$
d15 AR-MC3T3	$2.9 \pm 0.60$	$1446 \pm 86$	$5219 \pm 311$
d25 $\beta\text{gal}$ -MC3T3	$1.8 \pm 0.20$	$1308 \pm 469$	$4720 \pm 1691$
d25 AR-MC3T3	$2.6 \pm 0.20$	$2479 \pm 405$	$8947 \pm 1461$
Ventral prostate	$5.8 \pm 0.06$	$1351 \pm 85$	$4877 \pm 308$
LNCaP	$7.7 \pm 1.57$	$3971 \pm 598$	$14,335 \pm 2157$

Androgen receptor levels were quantitated by titration analysis using [ $^3\text{H}$ ] methyltrienolone ([ $^3\text{H}$ ]R1881). The DNA content was quantitated and specific [ $^3\text{H}$ ]R1881 binding capacity was estimated by one site binding with non-linear regression and Scatchard plot analysis. The specific binding of [ $^3\text{H}$ ]R1881 was expressed in fmol/mg DNA and sites/cell. Analysis by two-way ANOVA demonstrated that both cell line and time in culture significantly influenced AR levels.

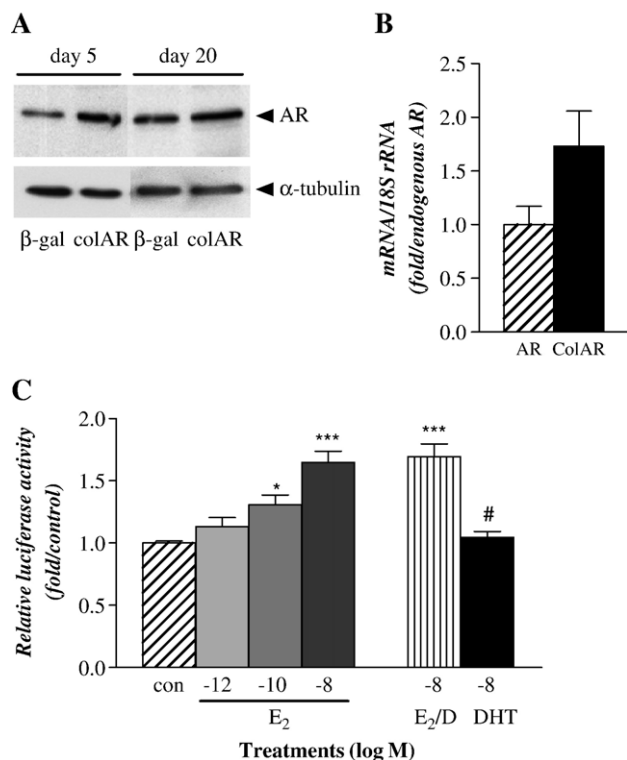


Fig. 1. Characterization of AR levels in AR-MC3T3 cultures during osteoblast differentiation. AR levels in AR-MC3T3 cultures during proliferation (day 5) and in mature osteoblasts/osteocytes (day 20) were assessed by Western analysis. (A) AR protein levels in stably transfected AR-MC3T3 and control  $\beta$ gal-MC3T3 cells. Both AR-MC3T3 and control  $\beta$ gal-MC3T3 cells were grown for the indicated time in culture, and AR abundance was determined in whole cell lysates with Western blot analysis using polyclonal rabbit antibodies recognizing AR (PA1-111A). The same membrane was reprobed with  $\alpha$ -tubulin antibodies as a loading control. The increase in AR levels with differentiation is also seen by Scatchard plot analysis presented in Table 1. (B) Characterization of colAR transgene gene expression by qRT-PCR analysis. Primers specific for the colAR transgene and for endogenous AR were used to evaluate relative AR transgene expression using total RNA isolated from AR-MC3T3 cultures. (C) Lack of effect of AR overexpression on transactivation of other steroid receptors. ER function was evaluated in AR-MC3T3 cultures in transient transfections using the ERE reporter construct ERE-luc. Cells were treated for 54 h with E<sub>2</sub> at  $10^{-12}$  M,  $10^{-10}$  M, and  $10^{-8}$  M in the presence or absence of  $10^{-8}$  M DHT. The data are expressed as the means  $\pm$  SEM ( $n = 6$ ) from two independent experiments. Analysis by ANOVA revealed significant differences ( $P < 0.0001$ ). Post hoc analysis using Newman–Keuls Multiple Comparison Test demonstrated a significant increase in reporter activity with E<sub>2</sub> treatment, but no effect of DHT alone or in the presence of E<sub>2</sub>. \* $P < 0.05$ ; \*\*\* $P < 0.001$  vs. control; # $P < 0.001$  vs. E<sub>2</sub> + DHT combined.

#### Characterization of osteoblast apoptosis: androgen and estrogen effects during proliferation

We have previously shown that continuous DHT treatment reduces osteoblast viability through reductions in MAP kinase signaling in both normal rat primary calvarial cells and in AR-MC3T3 cultures [57]. Since reductions in viability can be associated with enhanced apoptosis, we sought to determine whether DHT treatment also influenced osteoblast apoptosis. Three independent assays were employed to characterize apoptosis: nucleosome fragmentation, caspase-3 activity, and  $\Delta\psi_{\text{mito}}$  analysis. AR-MC3T3 cells were plated in 6-well dishes

and grown for 5 days in the continuous presence of either vehicle,  $10^{-8}$  M DHT, or  $10^{-8}$  M E<sub>2</sub>. Apoptosis was induced by treatment with 50  $\mu$ M etoposide for 18 h before harvest then assessed by oligonucleosome analysis (Fig. 2A) and with determination of caspase-3/CPP32 activity measured by substrate cleavage (Fig. 2B). DHT treatment significantly enhanced osteoblast apoptosis 1.5- to 2-fold, in the range described for well-characterized proapoptotic effects of glucocorticoid or TNF $\alpha$  (see [2,45]) and with BMP-2 administration (see [16]) in osteoblasts. Separate one-way ANOVAs for each treatment determined that both androgen and estrogen treatments were significantly different from control but with opposite effects: DHT treatment significantly enhanced while E<sub>2</sub> treatment inhibited apoptosis. Although not as robust presumably due to lower AR levels, DHT also stimulated apoptosis in control  $\beta$ gal-MC3T3 cultures (data not shown).

To extend these results, mitochondrial membrane permeability  $\Delta\psi_{\text{mito}}$  analysis was performed. AR-MC3T3 cells were rinsed with serum-free media, stained with MitoSensor Dye at 37°C for 20 min, and analyzed by fluorescence microscopy (Fig. 2C). Collapse of the electrochemical gradient across the mitochondrial membrane is an early indicator of the initiation of cellular apoptosis. MitoSensor (cationic dye) is taken up in the mitochondria where it forms red fluorescent aggregates in healthy cells. In apoptotic cells, the dye cannot aggregate because of altered  $\Delta\psi_{\text{mito}}$  and remains in a green fluorescent monomeric form in the cytoplasm. Modestly increased green/yellow staining with etoposide, enhanced with DHT treatment, is consistent with loss of mitochondrial membrane permeability and  $\Delta\psi$ . In contrast, red stained cells with E<sub>2</sub> treatment suggests that cultures are relatively protected from etoposide-induced apoptosis. In control cultures without etoposide induction, DHT and E<sub>2</sub> demonstrated similar but even less robust effects.

To establish whether androgen-induced apoptosis was mediated by functional AR, AR-MC3T3 cultures were treated with DHT in the presence of the specific non-steroidal AR antagonist hydroxyflutamide (OHF). As shown in Fig. 2D, an  $\sim 2.2$ -fold increase in apoptosis is observed after  $10^{-8}$  M DHT treatment for 5 days ( $P < 0.01$ ). However, coincubation with  $10^{-6}$  M OHF significantly abrogated the effect of DHT on apoptosis ( $P < 0.05$ ). OHF alone appeared to have a slight agonist effect as has been noted previously (see [56]); however, the effect was not significantly different from control.

#### Characterization of osteoblast apoptosis: androgen effects during differentiation in mature osteoblast/osteocyte cultures

Since much of the analysis of the effects of estrogen on osteoblast apoptosis has focused on osteocytic cells, known to demonstrate enhanced apoptosis [31], we next determined whether the effect of either estrogen or androgen treatment on osteocytic cells in extended culture at day 29 was similar to that observed in proliferating osteoblasts. We have shown that both ER $\alpha$  and ER $\beta$  mRNA and protein expression in osteoblastic cells were similar or elevated at days 25 or 30 when compared

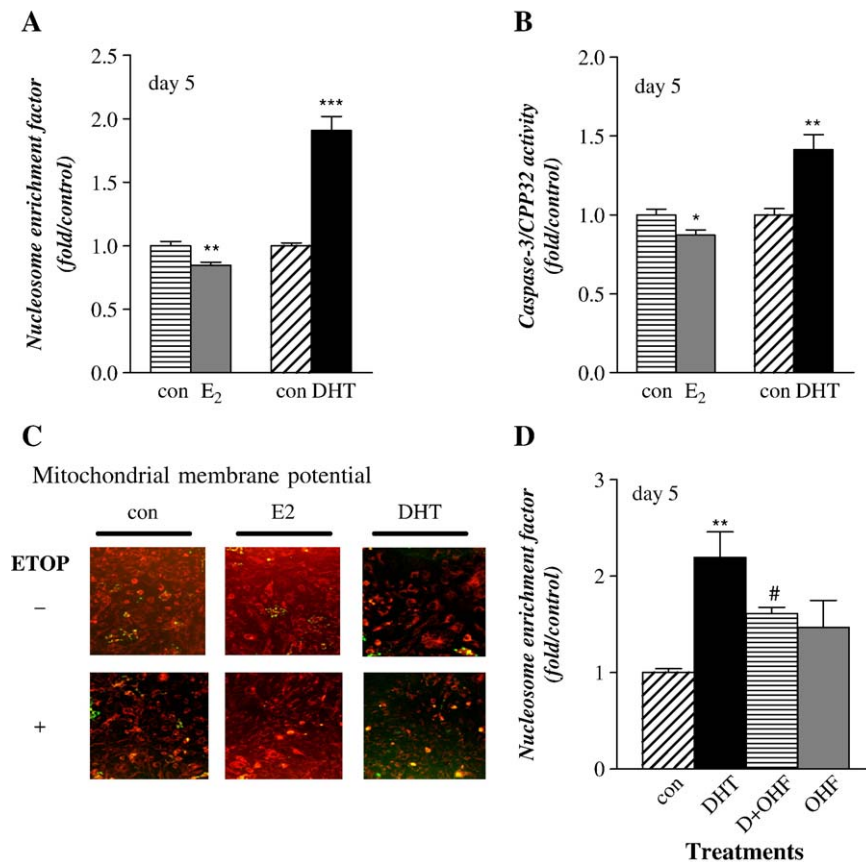


Fig. 2. Characterization of osteoblast apoptosis: results of androgen and estrogen treatment during proliferation. AR-MC3T3 cells were plated in 6-well dishes and grown for 5 days in the continuous presence of vehicle or  $10^{-8}$  M DHT or  $10^{-8}$  M E<sub>2</sub>. Apoptosis was induced by treatment with 50  $\mu$ M etoposide for 18 h before harvest. Apoptosis was characterized using three independent assays. (A) Cell Death oligonucleosome analysis. Apoptosis was characterized by analyzing DNA fragmentation with the Cell Death oligonucleosome assay. The data are presented as the nucleosome enrichment factor =  $A_{\text{treated}}/A_{\text{untreated}}$ , expressed as the means  $\pm$  SEM ( $n = 9$ –12) from three separate studies. Differences were statistically significant by Student's  $t$  test.  $**P < 0.01$ ;  $***P < 0.0001$  vs. control. (B) Caspase-3/CPP32 activity measured by substrate cleavage. Data are presented as mean  $\pm$  SEM ( $n = 8$ ). Experiments were carried out three times.  $*P < 0.05$ ;  $**P < 0.01$  vs. control. (C) Mitochondrial membrane potential ( $\Delta\Psi_{\text{mito}}$ ) analysis. AR-MC3T3 cells were rinsed with serum-free media, stained with MitoSensor Dye at 37°C for 20 min, and analyzed by fluorescence microscopy. All results are representative of at least three independent experiments. (D) Characterization of osteoblast apoptosis: dependence on AR function. AR-MC3T3 cells were cultured as described with  $10^{-8}$  M DHT treatment for 5 days with or without  $10^{-6}$  M hydroxyflutamide (OHF), an androgen receptor antagonist. Apoptosis was evaluated by Cell Death oligonucleosome analysis. The data are expressed as the means  $\pm$  SEM ( $n = 6$ ) from two independent experiments. Differences were statistically significant by one-way ANOVA ( $P = 0.0033$ ). Post hoc analysis with Newman–Keuls Multiple Comparison Test demonstrated that hydroxyflutamide significantly abrogates DHT-mediated apoptosis at day 5.  $**P < 0.01$  (vs. control);  $\#P < 0.05$  (vs. DHT).

with day 5 [55], and that AR-MC3T3 osteocytic cultures have enhanced AR expression (Fig. 1 and Table 1). Osteocytic cultures were characterized after continuous DHT and E<sub>2</sub> treatment (both at  $10^{-8}$  M) for 29 days (Fig. 3). Cultures were serum starved for the last 48 h in medium containing 0.1% bovine serum albumin. Apoptosis was induced in osteocytic cultures by serum starvation instead of etoposide administration since topoisomerase II activity (the target of etoposide treatment) is dramatically reduced in highly confluent cultures [47]. Quantitative analysis of apoptosis was determined using nucleosome fragmentation, caspase-3 activity, and mitochondrial membrane permeability as described in Fig. 2. Consistent with results in proliferating cultures, DHT enhanced but E<sub>2</sub> inhibited osteoblast apoptosis after continuous treatment in day 29 osteocytic cultures assessed by oligonucleosome analysis (Fig. 3A) and loss of mitochondrial membrane permeability (Fig. 3C). DHT treatment modestly increased caspase-3

activity but this result did not reach significance (Fig. 3B); however, E<sub>2</sub> did not suppress caspase-3 activity. These results may reflect the high levels of caspase-3 expression in osteocytic cultures in vitro [35].

#### DHT-mediated changes in key apoptotic regulators Bcl-2 and Bax

In order to characterize the mechanism underlying DHT-enhanced osteoblast apoptosis, we next determined the effect of androgen treatment on the important apoptotic regulators Bcl-2 and Bax. Western blot analysis was used to determine the levels of Bcl-2 and Bax protein in DHT- or E<sub>2</sub>-treated AR-MC3T3 cells (Fig. 4A). After DHT treatment, we observed significant enhancement of the Bax/Bcl-2 ratio by nearly 1.3-fold ( $P < 0.05$ ; Fig. 4B), which mostly reflected a decrease in Bcl-2 protein levels. E<sub>2</sub> treatment alone did not significantly alter the

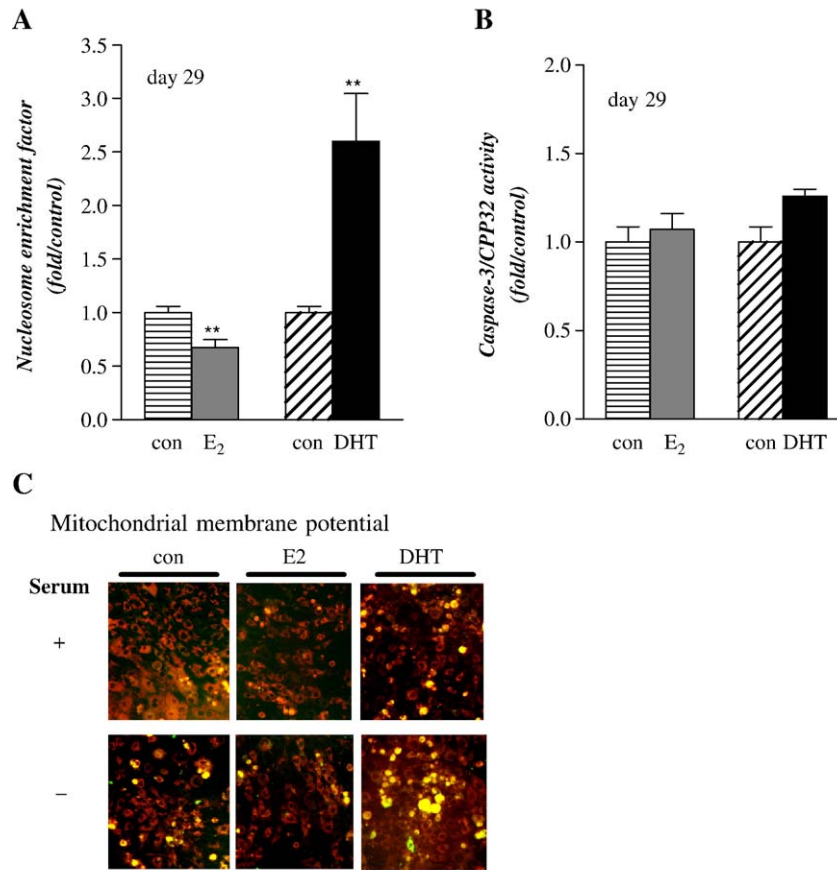


Fig. 3. Characterization of osteoblast apoptosis: androgen effects during differentiation in mature osteoblast/osteocyte cultures. Apoptosis was characterized in osteocyte-like cultures after continuous DHT and E<sub>2</sub> treatment (both at 10<sup>-8</sup> M) for 29 days. Apoptosis was induced by serum starvation for 48 h before isolation, replaced with 0.1% BSA. (A) Analysis of apoptosis evaluating DNA fragmentation analysis. The data are expressed as the means ± SEM (*n* = 6) from two independent experiments. See Fig. 2 for methods. \*\**P* < 0.01 vs. control. (B) Caspase-3/CPP-32 activity measured by substrate cleavage. Lysates from day 29 control and hormone-treated cultures were analyzed. The data are expressed as the means ± SEM (*n* = 6) from two independent experiments. (C) Mitochondrial membrane potential ( $\Delta\Psi_{\text{mito}}$ ) analysis. AR-MC3T3 cells were cultured with continuous DHT and E<sub>2</sub> treatment (both at 10<sup>-8</sup> M) for 29 days, stained with MitoSensor Dye, and analyzed by fluorescence microscopy. All results are representative of at least three independent experiments.

Bax/Bcl-2 ratio, consistent with previous analysis in osteoblasts [12]. To better characterize the time course of changes in Bcl-2 protein, Western analysis was performed after 24, 48, 72, and 96 h of continuous 10<sup>-8</sup> M DHT treatment (Fig. 4C). Quantitative analysis of Bcl-2 protein levels indicated by fold change showed that Bcl-2 levels were relatively unaffected for up to 48 h of DHT treatment, but then decreased by 72 h and 96 h of treatment to ~70% of control levels. This result is consistent with the time course observed for androgen-mediated changes in osteoblast viability [57].

We next investigated the mechanism by which androgen treatment stimulated the proapoptotic response by determining *bcl-2* and *bax* steady-state mRNA levels with real-time qRT-PCR analysis. AR-MC3T3 cells were incubated continuously with either DHT or E<sub>2</sub> (both at 10<sup>-8</sup> M) for 5 days and total RNA was isolated. Fig. 4D shows that *bax* mRNA levels were modestly elevated ~20% with DHT treatment, although this effect did not reach statistical significance. In contrast, *bcl-2* mRNA levels were not different compared to those for control vehicle-treated cells. Given the reductions in Bcl-2 protein but no corresponding decrease in *bcl-2* mRNA levels, these results

suggest that DHT regulation of Bcl-2 protein levels is post-transcriptional.

*The androgen-mediated reduction of Bcl-2 protein is associated with decreased Bcl-2 serine phosphorylation and increased ubiquitination*

MAP kinase activity has been shown to influence Bcl-2 protein degradation through changes in Bcl-2 phosphorylation [5,9]. Since we have previously demonstrated reduced MAP kinase signaling after chronic androgen treatment in osteoblastic cells [57], we sought to determine whether androgen treatment in osteoblasts also reduced Bcl-2 phosphorylation. AR-MC3T3 cells were treated with 10<sup>-8</sup> M DHT for 3 days and whole cell extracts were prepared. Equal amounts of protein were analyzed for the presence of total and phosphorylated Bcl-2 after immunoprecipitation followed with Western blot analysis using either pan Bcl-2 or antiphosphoserine-specific antibody (clone 4A9) for detection (Fig. 5A, left panel). DHT treatment reduced Bcl-2 protein to ~80% of control levels, with an additional reduction in serine phosphorylation to ~70% of

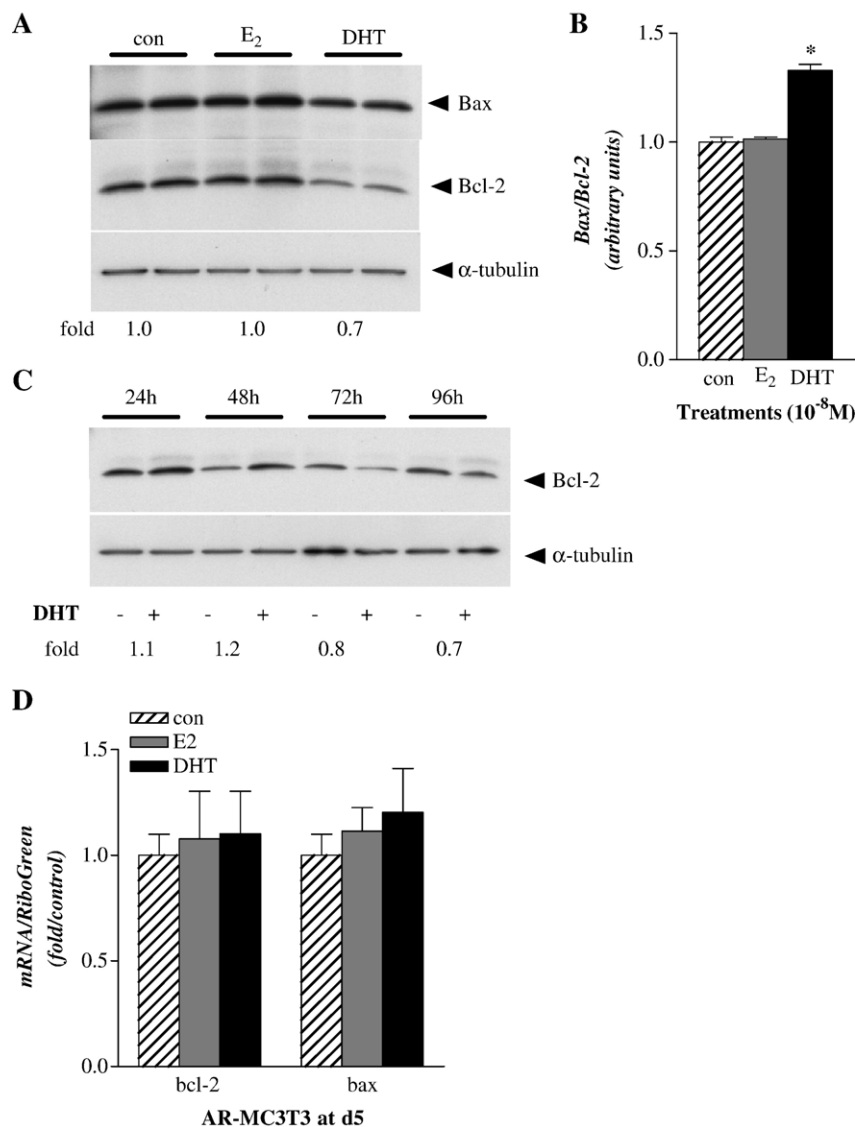


Fig. 4. DHT-mediated changes in key apoptotic regulators Bcl-2 and Bax. AR-MC3T3 cells were isolated at day 5 after continuous DHT or E<sub>2</sub> (10<sup>-8</sup> M) treatment. (A) Western blot analysis of Bcl-2 and Bax levels. Total cell lysates from AR-MC3T3 treated with steroids for 5 days were used to evaluate Bcl-2 and Bax protein levels. Extract was analyzed by 15% SDS-PAGE, transferred onto an Immobilon-P PVDF membrane and probed using polyclonal rabbit antibodies recognizing Bcl-2 or Bax as described in Materials and methods. Fold difference between control and DHT treatment is indicated for Bcl-2 levels. (B) Quantitative analysis of Bax/Bcl-2 ratio. Quantification of immunoband intensities from Western blot analysis as shown in panel A was determined by densitometric scanning. Data were expressed as Bax/Bcl-2 ratio. Results are representative of at least three independent experiments. \**P* < 0.05 vs. control. (C) Time course of Bcl-2 protein levels after androgen treatment. AR-MC3T3 cultures were treated with 10<sup>-8</sup> M DHT for 24, 48, 72, and 96 h, and Bcl-2 levels were characterized by Western blot analysis. Fold difference between control and DHT treatment is indicated for Bcl-2 levels. (D) Regulation of Bcl-2 is post-transcriptional. Levels of *bcl-2* and *bax* mRNA were analyzed by qRT-PCR analysis from total RNA isolated from control, E<sub>2</sub>, or DHT-treated AR-MC3T3 cultures for 5 days. Levels of *bcl-2* were unaffected by hormone treatment, while the levels of *bax* were modestly increased by DHT. The data are expressed as the means ± SEM (*n* = 4) from two experiments.

normal levels (Fig. 5A, right panel). To provide further evidence that proteasomes were involved in Bcl-2 degradation after androgen treatment, AR-MC3T3 cells were treated with DHT and the effect on ubiquitination of Bcl-2 was determined. Total Bcl-2 was isolated from whole cell lysates by immunoprecipitation followed by Western blot analysis with antiubiquitin antibody. As shown in Fig. 5B, ubiquitin conjugates of Bcl-2 were modestly increased by DHT treatment, consistent with post-transcriptional regulation. Combined, these results suggest that targeting of Bcl-2 for degradation by proteasomes is associated with reduced serine phosphorylation of Bcl-2 protein.

#### The apoptotic effect of DHT requires an increase in Bax/Bcl-2 ratio

In order to determine whether apoptosis stimulated by androgen treatment requires an increase in Bax/Bcl-2 ratio, expression of either *bcl-2* or *bax* mRNA was altered by transiently transfection. To reverse the inhibition of Bcl-2 expression by androgen treatment, Bcl-2 was overexpressed using the pCMV-Bcl-2 expression construct. AR-MC3T3 cells were transfected with pCMV-Bcl-2 or mock-transfected, then treated with DHT for a total of 48 h (30 h followed by

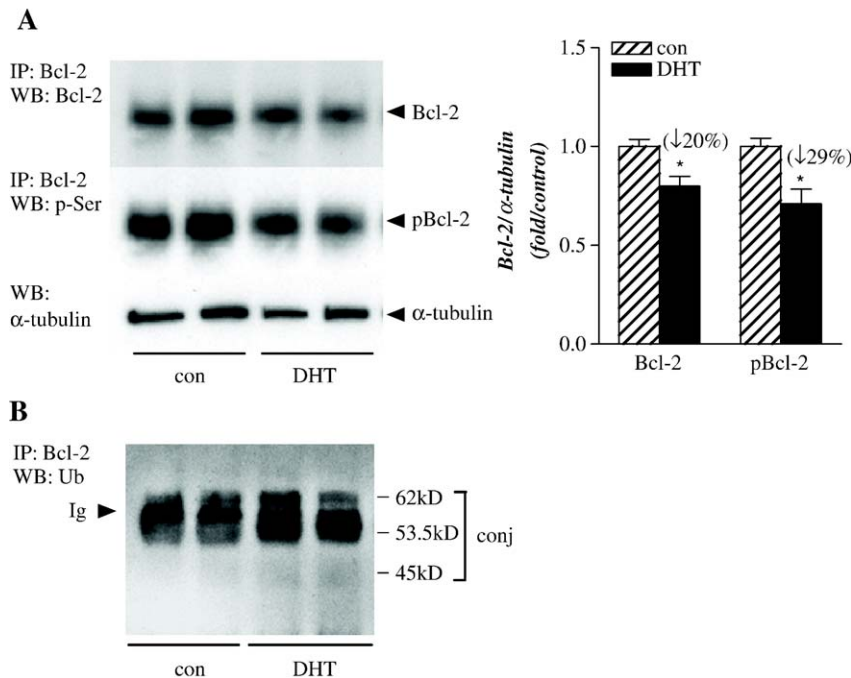


Fig. 5. The androgen-mediated reduction of Bcl-2 protein is associated with decreased Bcl-2 phosphorylation and proteasomal degradation. AR-MC3T3 cells were treated with  $10^{-8}$  M DHT for 72 h and equal amounts of protein were immunoprecipitated with anti-Bcl-2 antibody. Immunocomplexes were resolved by 12% SDS-PAGE and transferred onto Immobilon-P PVDF membrane. (A) Androgen treatment reduces Bcl-2 protein and induces Bcl-2 dephosphorylation. Western blot (WB) analysis was carried out with either anti-Bcl-2 antibody or antiphosphoserine-specific antibody after immunoprecipitation (IP). Equal protein loading was confirmed with  $\alpha$ -tubulin levels determined before immunoprecipitation by Western analysis from the same lysates. Quantification of immunoband intensities was determined by volumetric densitometric scanning (right panel). The data are expressed as the means  $\pm$  SEM ( $n = 4$ ) from two independent experiments.  $*P < 0.05$  vs. control. (B) Androgen induces Bcl-2 ubiquitination. Ubiquitin conjugates of Bcl-2 were identified with Western blot analysis using antiubiquitin antibody after immunoprecipitation. conj., conjugates; Ig, the heavy chain of the immunoglobulin molecule.

50  $\mu$ M etoposide for 18 h). Apoptosis was characterized at day 5 using mono- and oligonucleosome analysis as described in Fig. 2. In osteoblastic cultures with Bcl-2 overexpression (Fig. 6A), androgen-enhancement of apoptosis was completely reversed ( $P < 0.001$ ), confirming the importance of reductions in Bcl-2 levels. Overexpression of Bcl-2 protein was confirmed by Western analysis (Fig. 6C, left panel).

We next employed RNAi to inhibit Bax protein using an oligonucleotide-based technique with double-stranded siRNA validated for *bax*. AR-MC3T3 cells were transfected with siRNA Bax-specific oligonucleotides 24 h prior to the exposure of cells to DHT for 30 h, followed by 50  $\mu$ M etoposide for 18 h before isolation at day 5. As shown in Fig. 6B, DHT-mediated increases in osteoblast apoptosis were significantly abrogated with Bax inhibition ( $P < 0.001$ ). Western analysis of Bax protein levels in androgen treated AR-MC3T3 cells expressing Bax siRNA (Fig. 6C, right panel) confirmed that siRNA treatment resulted in a reduction in Bax protein expression to 60% of normal levels, with no effect after transfection with control siRNA oligonucleotides. A second source of *bax* siRNA oligonucleotides also abrogated DHT-mediated increases in osteoblast apoptosis (data not shown). These data suggest that an increase in the Bax/Bcl-2 ratio is important for androgen-mediated apoptosis, and is consistent with reports that Bcl-2 and Bax can function independently to regulate apoptosis [26].

#### *In vivo stimulation of osteoblast apoptosis in AR-transgenic mice*

In order to assess the physiological relevance of these observations, we characterized apoptosis in skeletally targeted AR-transgenic mice (for a description of the phenotype observed in these animals, please refer to reference [59]). AR-transgenic mice were created with the same full-length AR expression construct used for the AR-MC3T3 cells, demonstrate AR overexpression throughout the osteoblast lineage, and represent a model of enhanced responsiveness to circulating androgen without systemic hormone administration. An anabolic response to androgen in males was observed in H&E stained sections from calvaria isolated from male AR-transgenic mice vs. littermate controls at 2 months (Fig. 7A, left panels) and 6 months (Fig. 7A, right panels), and was not observed in females as previously reported [59]. Localization of new bone growth was characterized in calvarial cross sections using van Geison staining, where recently formed collagen is identified by a characteristic blue color [17]. As can be seen in Fig. 7B, new bone growth was present in calvaria as a result of AR-transactivation in post-pubertal (i.e., 2 month old; left panel) and adult (i.e., 6 month old; right panel) male AR-transgenic mice. There may be increased cell density in the woven bone present in the AR-transgenic male calvaria, consistent with the notion that woven bone has been estimated to contain 4 to 8 times more osteocytes than lamellar bone [18].

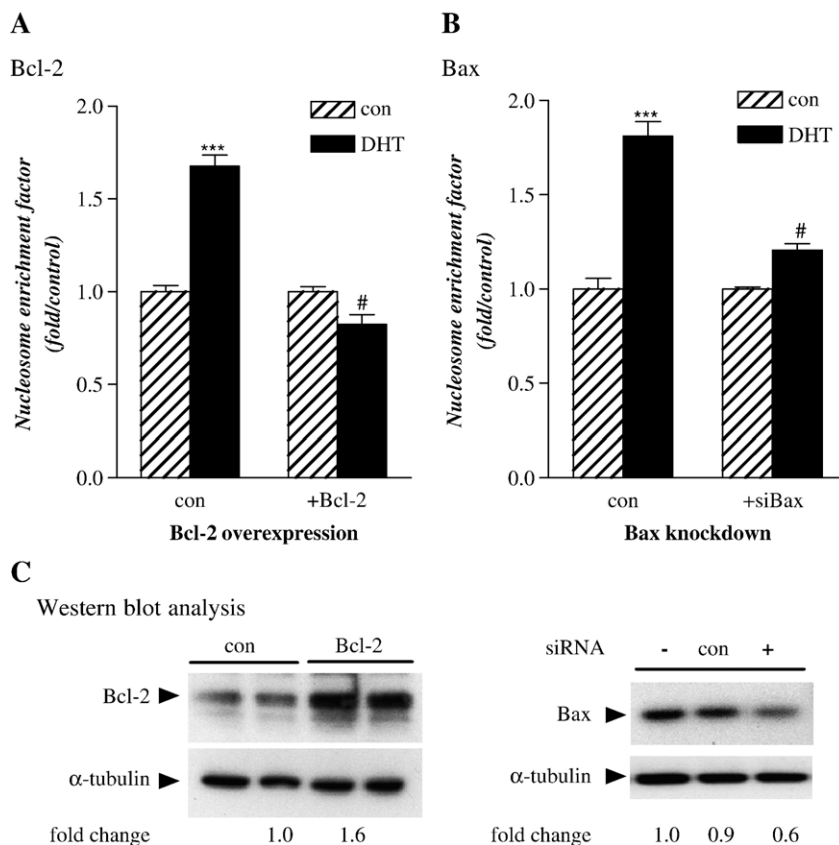


Fig. 6. The proapoptotic effect of DHT requires a decline in Bcl-2/Bax ratio. Bcl-2 or Bax levels were altered in AR-MC3T3 cultures after transient transfection. Cultures were transfected 1 day after plating and exposed to  $10^{-8}$  M DHT for 48 h. Apoptosis was induced by treatment with 50  $\mu$ M etoposide for 18 h before isolation at day 5. (A) Effect of Bcl-2 overexpression. Bcl-2 mRNA overexpression was achieved by transfection with expression construct pCMV-Bcl-2. Apoptosis was evaluated using the Cell Death oligonucleosome assay in transfected and mock-transfected cultures. The data are expressed as the mean  $\pm$  SEM ( $n = 6$ ) from two experiments. Differences were statistically significant by one-way ANOVA ( $P < 0.0001$ ). Post hoc analysis with Newman–Keuls Multiple Comparison Test demonstrated that overexpression of Bcl-2 completely reverses DHT-mediated apoptosis at day 5. \*\*\* $P < 0.001$  (vs. control); # $P < 0.001$  (vs. DHT control). (B) Effect of Bax knockdown with double-stranded siRNA treatment. Cultures were transfected with control siRNA (con) or Bax siRNA (double-stranded Bax siRNA) using the validated SureSilencing Mouse Bax siRNA kit as described in Materials and methods or were mock-transfected. Apoptosis was evaluated using the Cell Death oligonucleosome assay in transfected and control cultures. The data are expressed as the mean  $\pm$  SEM ( $n = 6$ ) from two experiments. Differences were statistically significant by one-way ANOVA. Post hoc analysis with Newman–Keuls Multiple Comparison Test demonstrated that inhibition of Bax ameliorates DHT-mediated apoptosis at day 5. \*\*\* $P < 0.001$  (vs. control); # $P < 0.001$  (vs. DHT control). (C) Western blot analysis for altered Bcl-2 and Bax expression. Proteins from total cellular extracts isolated 48 h after mock transfection or cultures transfected with Bcl-2 expression plasmid (left panel) or control or Bax siRNAs (right panel) were analyzed by Western blot probed with Bcl-2 or Bax antibody. Equal protein loading was confirmed by analysis with  $\alpha$ -tubulin antibody. Quantification of immunoband intensities was determined by densitometric scanning. The band intensity values of Bcl-2 or Bax were normalized with respect to the band intensity values of  $\alpha$ -tubulin. –, mock-transfection; con, control siRNA oligonucleotides; +, bax siRNA oligonucleotides.

Previously, characterization of the effects of androgen treatment on osteoblast apoptosis in vivo has been only performed in recently orchidectomized animals in a setting of high turnover and activated resorption [27], rather than associated with bone growth in an anabolic circumstance. We therefore characterized apoptosis in male AR-transgenic animals with TUNEL staining in calvarial cross sections. Post-pubertal (Fig. 7C, left panels) and adult (Fig. 7C, right panels) male AR-transgenic mice showed increased TUNEL staining when compared to wild-type controls, both in areas of new bone growth (proliferating osteoblasts). TUNEL staining was also demonstrated in matrix-embedded osteocytes in calvarial sections where new bone growth was not evident (Fig. 7D). Negative controls verified a lack of staining (see inset). Higher power magnifications of the apoptotic cells (see insets) identify the apoptotic cells as

osteoblastic and not multi-nucleated osteoclasts, based on location and morphology.

## Discussion

In this study, we explored the specific role of androgen, contrasted with estrogen, in the regulation of osteoblast apoptosis using in vivo and in vitro models of enhanced androgen responsiveness. We have previously demonstrated that chronic androgen treatment reduces osteoblast viability in both normal primary cultures and in clonal colAR-MC3T3 cultures, an effect mediated via inhibition of the ERK cascade [57]. Consistent with those findings, we report here that continuous treatment with the non-aromatizable androgen DHT stimulated, while similar treatment with  $E_2$  reduced, osteoblast apoptosis. Androgen treatment enhanced osteoblast apoptosis in

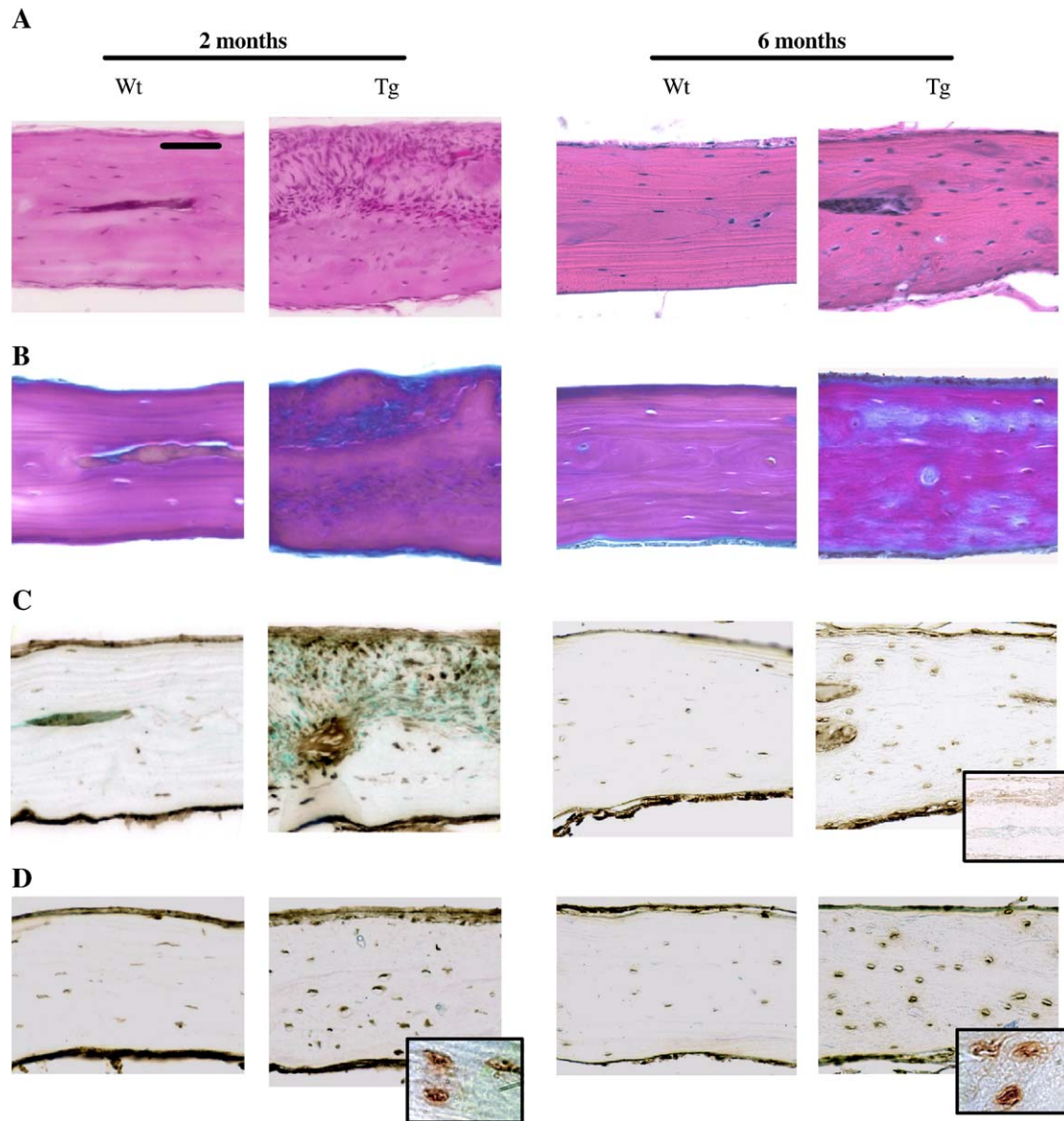


Fig. 7. Characterization of osteoblast apoptosis in vivo using AR-transgenic mice. Calvaria from either 2 month old (left panels) or 6 month old (right panels) male AR-transgenic or littermate control mice were fixed, decalcified, embedded, and calvarial cross sections were evaluated. (A) Calvarial histology. H&E stained cross sections show calvarial thickening in male AR-transgenic mice. Scale bar indicates 50  $\mu$ m. (B) New bone growth. New bone growth associated with new collagen synthesis was identified in calvarial cross sections using van Geison staining. New collagen synthesis is shown in post-pubertal 2 month old mice and in adult 6 month old mice. (C) Analysis of apoptosis with TUNEL staining with new bone formation. In situ end-labeling (TUNEL) staining was performed for analysis of apoptosis and counterstained with methyl green. Sections were derived from areas demonstrating new bone formation as described in panel C. Negative control sections for TUNEL analysis were incubated without TdT (inset). Quantitative analysis indicated  $\sim$ 10% apoptotic cells in wild-type and  $\sim$ 40% in AR-transgenic mice. (D) Analysis of apoptosis with TUNEL staining in osteocytic cells. TUNEL staining was performed in calvarial outer regions that do not demonstrate thickening. Enhanced apoptosis was observed in male AR-transgenic mice both in areas with new bone formation and in matrix-embedded osteocytes. Higher power magnification shows osteoblasts morphology (inset). wt, wild-type mice (left panels); tg, AR-transgenic mice (right panels). Microscope magnification at 40 $\times$  with higher power inset image at 100 $\times$ .

both proliferating and mature osteocyte-like cells in vitro, and the effects were abrogated in the presence of an androgen receptor antagonist. Levels of the antiapoptotic regulator Bcl-2 were reduced post-transcriptionally by androgen treatment, associated with enhanced ubiquitination. Reduced serine phosphorylation of Bcl-2 was also observed, consistent with inhibition of ERK1/2 activity. Notably, the increased Bax/Bcl-2 ratio was necessary for androgen-enhanced apoptosis since overexpression of *bcl-2* or siRNA knockdown of *bax* abrogated

the effects of DHT. In vivo analysis of calvarial sections from skeletally targeted male AR-transgenic mice at two time points also demonstrated enhanced TUNEL staining in osteoblasts when compared with wild-type controls, even in areas of new bone growth. Collectively, these data indicate that enhanced osteoblast apoptosis after androgen exposure is mediated by an increase in the Bax/Bcl-2 ratio via a reduction in Bcl-2 phosphorylation and MAP kinase activation, and is observed even in anabolic settings.

Notably, an association of osteoblast apoptosis with osteogenesis has been observed previously. During bone formation, osteoblasts undergo an orderly developmental progression ultimately ending in apoptosis [19]. It has been estimated that as many as 65% of osteoblasts undergo apoptosis after completing synthesis of bone matrix [25], suggesting that apoptosis is a fundamental component of the osteoblastic differentiation program [31]. TUNEL-positive osteoblasts and even preosteoblasts have been demonstrated at or close to the osteogenic fronts in areas of intense osteogenic activity during calvarial growth [40]. In addition, caspase-3-deficient mice show delayed ossification and decreased bone mineral density, suggesting that caspase activity plays a critical role in osteogenic differentiation in vivo [34]. Finally, bone-targeted Bcl-2 overexpression leads to reduced bone formation in a sex-specific fashion [38]. Thus, apoptosis can be associated with osteogenesis and the homeostatic maintenance of bone. This point is worth noting, since androgen-mediated apoptosis might be inappropriately viewed as inconsistent with anabolic actions.

In some tissues, androgen treatment has been reported to enhance apoptosis [54,64] and decrease Bcl-2 levels [6,20,30]. In this report, we show that treatment with DHT in osteoblasts also results in reduced Bcl-2 protein levels and enhanced apoptosis. Bcl-2 protein can be stabilized upon ERK1/2 activation in some settings [8,14,41]. While it has been reported that Bcl-2 phosphorylation can also facilitate apoptosis [60], it may also be that Bcl-2 phosphorylation can enhance survival but is not sufficient to prevent cell death with continuous toxin exposure [8]. Consistent with our studies, dephosphorylation of Bcl-2 results in enhanced apoptosis, mediated by ubiquitin-induced degradation of Bcl-2 [5,9]. These reports suggest a link between MAP kinase activity and the proteasome pathway to regulate Bcl-2 levels. Although three consensus MAP kinase sites have been identified in Bcl-2 [5], Bcl-2 is the target of multiple kinases including c-Jun N-terminal kinase 1, protein kinase A, and PKC $\alpha$  [8,23]. Furthermore, Bcl-2 phosphorylation is a dynamic process that is negatively regulated by protein phosphatase 2A [21]. In addition, the antiapoptotic effect of growth factor signaling may be mediated by additional proteins through PI3 kinase-mediated pathways [53]. Thus, it is likely that the regulation of the antiapoptotic function of Bcl-2 by phosphorylation is cell-type-specific and may be dependent on additional factors that are as yet undefined. Here, we demonstrate that, in osteoblasts, androgen treatment decreases Bcl-2 protein levels post-transcriptionally with reduced serine phosphorylation and increased ubiquitination. Thus, one mechanism through which androgen treatment enhances osteoblast apoptosis may be via inactivation of the antiapoptotic function of Bcl-2 by reducing Bcl-2 protein level through ubiquitin-mediated proteolytic degradation, as expected with suppressed ERK1/2 activity [57].

The antiapoptotic effect of estrogen and the proapoptotic effect of androgen treatment were observed both in proliferating osteoblasts and in mature osteocytic cultures. These results are supported by a recent publication of opposing effects of estrogen and androgen on apoptosis, here in T47D cells [22]. Further, our in vitro analyses are consistent with multiple

reports indicating that estrogen can reduce osteoblast apoptosis in some [12,27,65], but not all circumstances [11]. Neither Bcl-2 nor Bax expression was significantly influenced by E<sub>2</sub> treatment alone, contrary to the effects of androgen treatment. In the presence of apoptotic stimuli such as glucocorticoid treatment, E<sub>2</sub> is able to prevent the increased Bax/Bcl-2 ratio [12,13,38]. To our knowledge, this is the first report demonstrating divergent and distinct regulation by androgen vs. estrogen with regard to osteoblast apoptosis. These results again indicate that non-specific non-genomic signaling through either ERs or AR, reportedly activated with the same effectiveness regardless of whether the actual ligand is an androgen or an estrogen [27], is not a significant modulator of osteoblast apoptosis induced by androgen treatment. Analysis with AR overexpression in Fig. 1C is also consistent with mediation of androgen action strictly by AR transactivation, and not through non-specific interactions with ERs nor through generalized squelching of steroid receptor-mediated signaling.

A contrasting response to estrogen and androgen administration has also been described in some bone compartments in vivo. This is particularly true in cortical bone, where at the periosteum estrogen suppresses but androgen stimulates new bone formation [49], while at the endosteum estrogen stimulates but androgen strongly suppresses formation [59]. These envelope-specific responses likely play an important role in determining sexual dimorphism of the skeleton, i.e., that male bones are wider but not thicker than females [43]. In trabecular bone, ER and AR signaling may have functional redundancy, although not with exactly the same mechanism [36]. Combined, these results suggest that administration of both estrogen and androgen may be beneficial, and is consistent with clinical studies demonstrating that combination therapy with estrogen and androgen is more beneficial than either steroid alone in post-menopausal women [7,33], recently confirmed in an animal model [46]. Ongoing analysis examining the effects of both steroids in combination will be important for a better understanding of the impact of combination therapy on bone homeostasis.

Few studies have characterized the specific effect of androgen in vivo on osteoblast apoptosis. In one report, increased osteoblast apoptosis was seen after orchidectomy in adult mice (where both estrogen and androgen levels are reduced). Six weeks of DHT treatment in these orchidectomized mice was associated with reduced osteoblast apoptosis in vertebrae [27], a site of predominantly trabecular bone. However, in this setting soon after orchidectomy, bone resorption is dramatically increased and thus one cannot adequately evaluate a connection between anabolic effects of androgen treatment associated with new bone growth and osteoblast apoptosis. Results reported here are also in contrast to the in vitro response noted after short-term DHT treatment that demonstrated inhibition of apoptosis [27]. While the reasons for the discrepancy are not clear, inhibition of apoptosis reported in this setting may have been a consequence of stimulation of ERK activity associated with rapid non-genomic steroid actions, since longer androgen treatment results in reductions in MAP kinase activation [57]. Interestingly, it is possible that some

aspects of the anabolic response to androgen we and others have described could be the result of an increase in osteoblast cell proliferation via a transient non-genomic androgen-mediated increased MAP kinase, PKC or PI3 kinase, or other kinase cascades and/or calcium mobilization (e.g., see [24]), through potentiation of growth factor and/or cytokine signaling, or through an as yet unexplained mechanism. Androgen treatment has been reported to increase osteoblast proliferation in vitro when treatment times are short [57] or transient (e.g., 15 min, see [24]), and to increase collagen expression [3]. The anabolic response is likely to be complicated, however, since there is no clear mechanism to block androgen signaling after transient induction in vivo. In addition, osteogenesis in vivo is generally not associated with estrogen treatment that does increase MAP kinase activity [10].

In conclusion, we have demonstrated that chronic DHT treatment in both proliferating and in mature osteocytic cultures resulted in enhanced osteoblast apoptosis. This result contrasts with the inhibitory effects on apoptosis observed with E<sub>2</sub> treatment. An androgen-mediated increase in the Bax/Bcl-2 ratio was observed, predominantly through inhibition of Bcl-2. Increased Bax/Bcl-2 was necessary and sufficient for androgen-enhanced apoptosis since overexpression of *bcl-2* or RNAi knockdown of *bax* abrogated the effects of DHT, and was dependent on functional AR. These data suggest that enhanced apoptosis is mediated by an increase in the Bax/Bcl-2 ratio, at least in part as a consequence of reductions in Bcl-2 phosphorylation and protein stability consistent with inhibition of MAP kinase pathway activation. Analysis of calvaria in AR-transgenic male mice demonstrated enhanced TUNEL staining in vivo even in areas of new bone growth. In bone, apoptosis in osteoblasts has been reported in vitro and during development in vivo [31]. As has been observed in other remodeling tissues and/or associated with development and tissue homeostasis [29], mounting evidence has identified an association between new bone growth and apoptosis [37]. Apoptotic cell death could thus be important in making room for new bone formation and matrix deposition, which may have clinical significance by influencing bone homeostasis and bone mineral density [34]. Collectively, the findings reported here help to resolve a controversy surrounding the role of sex steroids in bone homeostasis by demonstrating that androgen directly stimulates while estrogen inhibits osteoblast apoptosis, and suggests that modulation of osteoblast apoptosis may be an important consequence of both androgen and estrogen signaling but with distinct outcomes. These data demonstrate that androgen signaling through the AR in bone directly influences osteoblast function in growing tissue in vivo and offer valuable insight into the role of androgen signaling in bone homeostasis. Further studies on androgen action in the developing skeleton, during repair and in the adult are warranted.

## Acknowledgments

The authors wish to thank Betsey Ramsey for assistance with Scatchard analysis and Dr. Louis Gerstenfeld (Boston Medical

Center) for advice regarding staining of new bone formation. We would also like to thank Dr. Stanley Korsmeyer (Harvard Medical School) for providing the pCMV-Bcl-2 expression construct, Dr. David Shapiro (University of Illinois) for the 4ERE-TATA-luc reporter construct, and Drs. Karl Jepsen and Mitch Schaffler (Mt. Sinai School of Medicine) for helpful discussions. This work is funded by Veterans Affairs Medical Research Service Merit Review program and the United States Army Research Acquisition Activity Award No. W81XWH-05-1-0086.

## References

- [1] Abud H. Shaping developing tissues by apoptosis. *Cell Death Differ* 2004;11:797–9.
- [2] Ahuja S, Zhao S, Bellido T, Plotkin L, Jimenez F, Bonewald L. CD40 ligand blocks apoptosis induced by tumor necrosis factor alpha, glucocorticoids, and etoposide in osteoblasts and the osteocyte-like cell line murine long bone osteocyte-Y4. *Endocrinology* 2003;144:1761–9.
- [3] Benz D, Haussler M, Thomas M, Speelman B, Komm B. High-affinity androgen binding and androgenic regulation of  $\alpha 1(I)$ -procollagen and transforming growth factor- $\beta$  steady state messenger ribonucleic acid levels in human osteoblast-like osteosarcoma cells. *Endocrinology* 1991;128:2723–30.
- [4] Bras M, Queenan B, Susin S. Programmed cell death via mitochondria: different modes of dying. *Biochemistry (Mosc)* 2005;70:231–9.
- [5] Breitschopf K, Haendeler J, Malchow P, Zeiher A, Dimmeler S. Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degradation: molecular characterization of the involved signaling pathway. *Mol Cell Biol* 2000;20:1886–96.
- [6] Bruckheimer E, Spurgers K, Weigel N, Logothetis C, McDonnell T. Regulation of Bcl-2 expression by dihydrotestosterone in hormone sensitive LNCaP-FGC prostate cancer cells. *J Urol* 2003;169:1553–7.
- [7] Castelo-Branco C, Vicente J, Figueras F, Sanjuan A, Martinez de Osaba M, Casals E, et al. Comparative effects of estrogens plus androgens and tibolone on bone, lipid pattern and sexuality in postmenopausal women. *Maturitas* 2000;34:161–8.
- [8] Deng X, Gao F, T. Flagg, May Jr WS. Mono- and multisite phosphorylation enhances Bcl2's antiapoptotic function and inhibition of cell cycle entry functions. *Proc Natl Acad Sci U S A* 2004;101:153–8.
- [9] Dimmeler S, Breitschopf K, Haendeler J, Zeiher A. Dephosphorylation targets Bcl-2 for ubiquitin-dependent degradation: a link between the apoptosome and the proteasome pathway. *J Exp Med* 1999;189:1815–22.
- [10] Endoh H, Sasaki H, Maruyama K, Takeyama K, Waga I, Shimizu T, et al. Rapid activation of MAP kinase by estrogen in the bone cell line. *Biochem Biophys Res Commun* 1997;235:99–102.
- [11] Garcia-Moreno C, Catalan M, Ortiz A, Alvarez L, De La Piedra C. Modulation of survival in osteoblasts from postmenopausal women. *Bone* 2004;35:170–7.
- [12] Gohel A, McCarthy M, Gronowicz G. Estrogen prevents glucocorticoid-induced apoptosis in osteoblasts in vivo and in vitro. *Endocrinology* 1999;140:5339–47.
- [13] Gu G, Hentunen T, Nars M, Harkonen P, Vaananen H. Estrogen protects primary osteocytes against glucocorticoid-induced apoptosis. *Apoptosis* 2005;10:583–95.
- [14] Hahn M, Li W, Yu C, Rahmani M, Dent P, Grant S. Rapamycin and UCN-01 synergistically induce apoptosis in human leukemia cells through a process that is regulated by the Raf-1/MEK/ERK, Akt, and JNK signal transduction pathways. *Mol Cancer Ther* 2005;4:457–70.
- [15] Hashimoto J, Beadles-Bohling A, Wiren K. Comparison of RiboGreen and 18S rRNA quantitation for normalizing real-time RT-PCR expression analysis. *BioTechniques* 2004;36:54–60.
- [16] Hay E, Lemonnier J, Fromigie O, Marie P. Bone morphogenetic protein-2 promotes osteoblast apoptosis through a Smad-independent, protein kinase C-dependent signaling pathway. *J Biol Chem* 2001;276:29028–36.

- [17] He H, Liu R, Desta T, Leone C, Gerstenfeld L, Graves D. Diabetes causes decreased osteoclastogenesis, reduced bone formation, and enhanced apoptosis of osteoblastic cells in bacteria stimulated bone loss. *Endocrinology* 2004;145:447–52.
- [18] Hernandez C, Majeska R, Schaffler M. Osteocyte density in woven bone. *Bone* 2004;35:1095–9.
- [19] Hock J, Krishnan V, Onyia J, Bidwell J, Milas J, Stanislaus D. Osteoblast apoptosis and bone turnover. *J Bone Miner Res* 2001;16:975–84.
- [20] Huang H, Zegar-Moro O, Benson D, Tindall D. Androgens repress Bcl-2 expression via activation of the retinoblastoma (RB) protein in prostate cancer cells. *Oncogene* 2004;23:2161–76.
- [21] Jiffar T, Kurinna S, Suck G, Carlson-Bremer D, Ricciardi M, Konopleva M, et al. PKC alpha mediates chemoresistance in acute lymphoblastic leukemia through effects on Bcl2 phosphorylation. *Leukemia* 2004;18:505–12.
- [22] Kampa M, Nifli A, Charalampopoulos I, Alexaki V, Theodoropoulos P, Stathopoulos E, et al. Opposing effects of estradiol- and testosterone-membrane binding sites on T47D breast cancer cell apoptosis. *Exp Cell Res* 2005;307:41–51.
- [23] Kanda H, Miura M. Regulatory roles of JNK in programmed cell death. *J Biochem (Tokyo)* 2004;136:1–6.
- [24] Kang H, Cho C, Huang K, Wang J, Hu Y, Lin H, et al. Nongenomic androgen activation of phosphatidylinositol 3-kinase/Akt signaling pathway in MC3T3-E1 osteoblasts. *J Bone Miner Res* 2004;19:1181–90.
- [25] Katavic V, Lukic I, Kovacic N, Grcevic D, Lorenzo J, Marusic A. Increased bone mass is a part of the generalized lymphoproliferative disorder phenotype in the mouse. *J Immunol* 2003;170:1540–7.
- [26] Knudson C, Korsmeyer S. Bcl-2 and Bax function independently to regulate cell death. *Nat Genet* 1997;16:358–63.
- [27] Kousteni S, Chen J, Bellido T, Han L, Ali A, O'Brien C, et al. Reversal of bone loss in mice by nongenotropic signaling of sex steroids. *Science* 2002;298:843–6.
- [28] Kousteni S, Han L, Chen J, Almeida M, Plotkin L, Bellido T, et al. Kinase-mediated regulation of common transcription factors accounts for the bone-protective effects of sex steroids. *J Clin Invest* 2003;111:1651–64.
- [29] Lanz R, Chua S, Barron N, Soder B, DeMayo F, O'Malley B. Steroid receptor RNA activator stimulates proliferation as well as apoptosis in vivo. *Mol Cell Biol* 2003;23:7163–76.
- [30] Ling S, Dai A, Williams M, Myles K, Dilley R, Komesaroff P, et al. Testosterone (T) enhances apoptosis-related damage in human vascular endothelial cells. *Endocrinology* 2002;143:1119–25.
- [31] Lynch M, Capparelli C, Stein J, Stein G, Lian J. Apoptosis during bone-like tissue development in vitro. *J Cell Biochem* 1998;68:31–49.
- [32] Marcus R, Leary D, Schneider D, Shane E, Favus M, Quigley C. The contribution of testosterone to skeletal development and maintenance: lessons from the androgen insensitivity syndrome. *J Clin Endocrinol Metab* 2000;85:1032–7.
- [33] Miller B, De Souza M, Slade K, Luciano A. Sublingual administration of micronized estradiol and progesterone, with and without micronized testosterone: effect on biochemical markers of bone metabolism and bone mineral density. *Menopause* 2000;7:318–26.
- [34] Miura M, Chen X, Allen M, Bi Y, Gronthos S, Seo B, et al. A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells. *J Clin Invest* 2004;114:704–13.
- [35] Mogi M, Togari A. Activation of caspases is required for osteoblastic differentiation. *J Biol Chem* 2003;278:47477–82.
- [36] Moverare S, Venken K, Eriksson A, Andersson N, Skrtic S, Wergedal J, et al. Differential effects on bone of estrogen receptor alpha and androgen receptor activation in orchidectomized adult male mice. *Proc Natl Acad Sci U S A* 2003;100:13573–8.
- [37] Palumbo C, Ferretti M, De Pol A. Apoptosis during intramembranous ossification. *J Anat* 2003;203:589–98.
- [38] Pantschenko A, Zhang W, Nahounou M, McCarthy M, Stover M, Lichtler A, et al. Effect of osteoblast-targeted expression of bcl-2 in bone: differential response in male and female mice. *J Bone Miner Res* 2005;20:1414–29.
- [39] Plotkin L, Aguirre J, Kousteni S, Manolagas S, Bellido T. Bisphosphonates and estrogens inhibit osteocyte apoptosis via distinct molecular mechanisms downstream of extracellular signal-regulated kinase activation. *J Biol Chem* 2005;280:7317–25.
- [40] Rice D, Kim H, Thesleff I. Apoptosis in murine calvarial bone and suture development. *Eur J Oral Sci* 1999;107:265–75.
- [41] Sarker K, Biswas K, Rosales J, Yamaji K, Hashiguchi T, Lee K. Ebselen inhibits NO-induced apoptosis of differentiated PC12 cells via inhibition of ASK1-p38 MAPK-p53 and JNK signaling and activation of p44/42 MAPK and Bcl-2. *J Neurochem* 2003;v:1345–53.
- [42] Sato T, Kawano H, Kato S. Study of androgen action in bone by analysis of androgen-receptor deficient mice. *J Bone Miner Metab* 2002;20:326–30.
- [43] Seeman E. The structural and biomechanical basis of the gain and loss of bone strength in women and men. *Endocrinol Metab Clin North Am* 2003;32:25–38.
- [44] Sims N, Clement-Lacroix P, Minet D, Fraslon-Vanhulle C, Gaillard-Kelly M, Resche-Rigon M, et al. A functional androgen receptor is not sufficient to allow estradiol to protect bone after gonadectomy in estradiol receptor-deficient mice. *J Clin Invest* 2003;111:1319–27.
- [45] Sowa H, Kaji H, Iu M, Tsukamoto T, Sugimoto T, Chihara K. Parathyroid hormone-Smad3 axis exerts anti-apoptotic action and augments anabolic action of transforming growth factor beta in osteoblasts. *J Biol Chem* 2003;278:52240–52.
- [46] Tivesten A, Moverare-Skrtic S, Chagin A, Venken K, Salmon P, Vanderschueren D, et al. Additive protective effects of estrogen and androgen treatment on trabecular bone in ovariectomized rats. *J Bone Miner Res* 2004;19:1833–9.
- [47] Tolner B, Hartley J, Hochhauser D. Transcriptional regulation of topoisomerase II alpha at confluence and pharmacological modulation of expression by bis-benzimidazole drugs. *Mol Pharmacol* 2001;59:699–706.
- [48] Tomkinson A, Reeve J, Shaw R, Noble B. The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. *J Clin Endocrinol Metab* 1997;82:3128–35.
- [49] Turner R, Wakley G, Hannon K. Differential effects of androgens on cortical bone histomorphometry in gonadectomized male and female rats. *J Orthop Res* 1990;8:612–7.
- [50] van der Eerden B, Emons J, Ahmed S, van Essen H, Lowik C, Wit J, et al. Evidence for genomic and nongenomic actions of estrogen in growth plate regulation in female and male rats at the onset of sexual maturation. *J Endocrinol* 2002;175:277–88.
- [51] Vandenput L, Swinnen J, Boonen S, Van Herck E, Erben R, Bouillon R, et al. Role of the androgen receptor in skeletal homeostasis: the androgen-resistant testicular feminized male mouse model. *J Bone Miner Res* 2004;19:1462–70.
- [52] Vanderschueren D, Vandenput L, Boonen S, Lindberg M, Bouillon R, Ohlsson C. Androgens and bone. *Endocr Rev* 2004;25:389–425.
- [53] Vantler M, Caglayan E, Zimmermann W, Baumer A, Rosenkranz S. Systematic evaluation of anti-apoptotic growth factor signaling in vascular smooth muscle cells: only phosphatidylinositol 3'-kinase is important. *J Biol Chem* 2005;280:14168–76.
- [54] Verzola D, Gandolfo M, Salvatore F, Villaggio B, Gianiorio F, Traverso P, et al. Testosterone promotes apoptotic damage in human renal tubular cells. *Kidney Int* 2004;65:1252–61.
- [55] Wiren K, Chapman Evans A, Zhang X. Osteoblast differentiation influences androgen and estrogen receptor-alpha and -beta expression. *J Endocrinol* 2002;175:683–94.
- [56] Wiren K, Keenan E, Zhang X, Ramsey B, Orwoll E. Homologous androgen receptor up-regulation in osteoblastic cells may be associated with enhanced functional androgen responsiveness. *Endocrinology* 1999;140:3114–24.
- [57] Wiren K, Toombs A, Zhang X-W. Androgen inhibition of MAP kinase pathway and Elk-1 activation in proliferating osteoblasts. *J Mol Endocrinol* 2004;32:209–26.
- [58] Wiren K, Zhang X-W, Chang C, Keenan E, Orwoll E. Transcriptional up-regulation of the human androgen receptor by androgen in bone cells. *Endocrinology* 1997;138:2291–300.
- [59] Wiren K, Zhang X-W, Toombs A, Gentile M, Kasparcova V, Harada S-I, et

- al. Targeted overexpression of androgen receptor in osteoblasts: unexpected complex bone phenotype in growing animals. *Endocrinology* 2004;145:3507–22.
- [60] Yamamoto K, Ichijo H, Korsmeyer S. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. *Mol Cell Biol* 1999;19:8469–78.
- [61] Yang W, Lu Y, Kalajzic I, Guo D, Harris M, Gluhak-Heinrich J, et al. Dentin matrix protein 1 gene cis-regulation: use in osteocytes to characterize local responses to mechanical loading in vitro and in vivo. *J Biol Chem* 2005;280:20680–90.
- [62] Yeh S, Tsai M, Xu Q, Mu X, Lardy H, Huang K, et al. Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. *Proc Natl Acad Sci U S A* 2002;99:13498–503.
- [63] Zagar Y, Chaumaz G, Lieberherr M. Signaling cross-talk from Gbeta4 subunit to Elk-1 in the rapid action of androgens. *J Biol Chem* 2004;279:2403–13.
- [64] Zhang H, Zhu Z, Liu L, Guo D, Jiang T, Wang L, et al. Upregulation of Fas and FasL expression in testosterone-induced apoptosis of macrophages. *Methods Find Exp Clin Pharmacol* 2003;25:779–84.
- [65] Zhou S, Zilberman Y, Wassermann K, Bain S, Sadovsky Y, Gazit D. Estrogen modulates estrogen receptor alpha and beta expression, osteogenic activity, and apoptosis in mesenchymal stem cells (MSCs) of osteoporotic mice. *J Cell Biochem* 2001;81:144–55.

Appendix 2 follows:

Bi LX, Wiren KM, Zhang X-W, Oliveira GV, Klein GL, Mainous EG, Herndon DN: Direct effect of oxandrolone treatment on human osteoblastic cells is modest. *J Burns and Wounds* *in review*, 2006

# **Direct Effect Of Oxandrolone Treatment On Human Osteoblastic Cells is Modest**

**Lian Xiang Bi 1,4,6, Kristine M. Wiren<sup>7,8</sup>, Xiao-Wei Zhang<sup>7,8</sup>, Gisele V. Oliveira 3,5,6,  
Gordon L. Klein 2,6, Elgene G. Mainous 4, David N. Herndon 3,6**

**Departments of Oral and Maxillofacial Surgery 1, Pediatrics 2, Surgery 3, Orthopaedic  
Surgery 4, and Dermatology 5 University of Texas Medical Branch and Shriners Burns  
Hospital 6, Galveston TX USA**

**Research Service<sup>7</sup>, VA Medical Center, and Departments of Medicine and Behavioral  
Neuroscience<sup>8</sup>, Oregon Health & Science University, Portland OR 97239**

**Correspondence to:**

**LX Bi MD**

**Department of Orthopaedic Surgery**

**2.804 Rebecca Sealy Hospital**

**University of Texas Medical Branch**

**301 University Boulevard**

**Galveston TX 77555-0892 USA**

**Phone: (409) 747-3204**

**Fax: (409) 747-3240**

**e-mail: [lbi@utmb.edu](mailto:lbi@utmb.edu)**

## Abstract

The anabolic agent oxandrolone, when given to severely burned children during the first year post-burn, produces an increase in lean body mass by six months but an increase in total body bone mineral content requires 12 months. Because of this relative delay, the bone mineral response may be dependent on the increased muscle mass causing an increased skeletal load. Alternatively, oxandrolone may act directly on bone. The aim of our study was to determine whether oxandrolone was able to transactivate the androgen receptor in osteoblasts. Collagen, alkaline phosphatase, osteocalcin, osteoprotegerin and androgen receptor abundance were determined by qRT-PCR, confocal laser scanning microscopy or immunoquantitative assay. In initial studies, proliferating human osteoblastic cells derived by collagenase digestion from bone fragments were stimulated with oxandrolone at doses ranging from 500 ng /ml to 30 µg/ml for 24 hours. Increased nuclear fluorescence of the androgen receptor and increased cellular type I collagen was observed with oxandrolone stimulation at 15 and 30 µg/ml but not at lower doses. Alkaline phosphatase (7-20%) and osteocalcin (13-18%) increases were modest but significant. To determine the effect of oxandrolone on gene expression in differentiated cells, osteocytic cultures grown to confluence in differentiation medium and then treated with 15 µg/ml oxandrolone for 24 hours or 5 days. No significant effects were observed with short-term treatment, but at 5 days androgen receptor levels were increased while collagen levels were decreased significantly with little effect on alkaline phosphatase, osteocalcin or osteoprotegerin. These data suggest that oxandrolone can directly stimulate production of osteoblast differentiation markers in proliferating osteoblastic cells most likely by means of the androgen receptor, but with longer treatment in mature cells, oxandrolone treatment decreases collagen expression. Thus it is possible that oxandrolone given to burned children acts directly on

immature osteoblasts to stimulate increased collagen production, but also may have positive effects to increase bone mineral content through other mechanisms.

## Introduction

Long-term use of the orally-administered anabolic agent oxandrolone has been shown to increase both lean body mass and bone mineral content in severely burned children when given over the first year post-burn (1). Oxandrolone and recombinant human growth hormone (2) were shown to be effective in recovering bone that would ordinarily be lost following the burn injury, resulting in markedly reduced bone formation (3) hypocellularity at the mineralization front of bone (3,4) and decreased marrow stromal cell differentiation into osteoblasts (4).

Increased endogenous glucocorticoid production is likely responsible for the acute bone loss observed in severely burned patients (3,4). Notably, with both oxandrolone and recombinant human growth hormone, an increase in lean body mass precedes an increase in bone mineral content by 3-6 months (1, 2). Thus it is not clear whether these anabolic agents increase bone mineral content secondary to increased muscle mass and hence increased skeletal loading, or whether they act directly on osteoblastic cells. Oxandrolone is an anabolic steroid with the ability to transactivate the androgen receptor (AR), which may be one mechanism underlying the anabolic response to therapy. The aim of our study is to determine whether oxandrolone can increase osteoblastic production of type I collagen and whether this action is mediated by AR signaling.

## Materials and Methods

*Human Osteoblast Cell Cultures:* Freshly discarded human cancellous bones were obtained from healthy young patients undergoing osteotomy. The bone fragments were washed with serum-free  $\alpha$ -minimum essential medium ( $\alpha$ -MEM, Flow Laboratories, McLean, VA). Then, the fragments were digested in the medium with 1 mg/ml collagenase for 2 hr at 37° C. The enzymatic reaction was stopped by adding an equal volume of  $\alpha$ -MEM with 10% fetal bovine serum (FBS). The supernatant containing the released cells was recovered. Washing and recovering were repeated three times. The cells were transferred to a centrifuge tube and centrifuge 10 min at 100xg to harvest the cells. The cell pellet was resuspended in 5 ml of fresh medium. The single cell suspension was cultured in  $\alpha$ -MEM containing antibiotics [penicillin (100U/ml), streptomycin sulphate (100ug/ml)] and 10% FBS in a humidified incubator at 37° C under an atmosphere of 5% CO<sub>2</sub> and 95% air. After confluence, adherent cells were collected by trypsinizing with 0.025% trypsin-EDTA and resuspended in  $\alpha$ -MEM. Only passages 4-8 were used in this study. Medium was changed every other day (4).

*Osteocytic cell culture:* Osteoblasts *in vitro* progress through several developmental stages that correlate with osteoblast development *in vivo*: from committed preosteoblasts; to mature, differentiated osteoblasts; and finally to osteocyte-like cells embedded in mineralized extracellular matrix (7-9). To characterize the effects of oxandrolone in osteocytic cells, human osteoblast cells were cultured in  $\alpha$ -MEM with 5% fetal bovine serum containing antibiotics for 10 d until confluence, then switched to BGJb medium containing 50  $\mu$ g/ml ascorbic acid and 3 mM  $\beta$ -glycerolphosphate (9). Cultures were maintained in differentiation medium for 7 d for mature osteoblast/osteocyte development (9), then treated with oxandrolone was initiated for

time course and dose response analysis. These highly confluent osteocytic cultures were treated in 5% charcoal-stripped serum with either 15 µg/ml of oxandrolone for 24 h or 5 d, or with increasing concentrations of oxandrolone (0, 1, 5, 10 and 15 µg/ml) for 5 d. Total RNA was isolated for gene expression analysis as described below.

*Immunohistochemistry for collagen type I and AR:* Immunohistochemical staining was carried out for both the AR (5,6) and type I collagen. Human osteoblastic cells were trypsinized, seeded on slide chambers (Lab-Tek, Nalge Nunc International, Rochester, NY) at a concentration of  $1 \times 10^5$  cells/ml and cultured for 3 d until they achieved 60-70% confluence. Oxandrolone was used to stimulate the cells at the concentration of 1, 5, 10, 15 and 30 µg/ml for 24 h. Stock solution containing 2.5 mg diluted in 2 ml of DMSO was prepared and subsequently diluted in culture media, while control non-stimulated cells received the same solution without oxandrolone. Thereafter, the medium was discarded, the cells were washed with PBS and fixed in minus 20° C Methanol/Acetone (50:50) for 20 min and permeabilized with 0.5% Triton X-100 (Sigma, Saint Louis, USA). The polyclonal antibody used for collagen I was applied overnight at the dilution of 1:200 (Research Diagnostics Inc.®, Flanders, NJ). The polyclonal antibody for AR (Neomarkers, Fremont, USA) was used at the dilution of 1:30. The next day, FITC-conjugated goat anti-rabbit IgG was used as secondary antibody (Neomarkers®, Fremont, CA) and cell nuclei were stained with 4,6-Diamidino-2-phenylindole (DAPI) (Vector Mounting Medium®, Burlingame, CA). Images were captured using the laser confocal scanning microscope (Zeiss LSM 510®, Jena, Germany) with a 63X objective. Optical sections were obtained from the cells by capturing single images of central cell focal planes. Three microscopic fields were captured for the control group of each cell line and the same was done

for cells treated with oxandrolone. In order to proceed to an analysis of AR immunohistochemistry, the intensity of fluorescence was measured in the nuclei (AR) in two cells per field of 3 fields, using Image Tool software (The University of Texas Health Science Center, San Antonio TX).

*Assay of alkaline phosphatase:* Human osteoblastic cells were cultured in  $\alpha$ -MEM containing antibiotics and 10% fetal bovine serum (FBS). The cells were challenged with increasing concentrations of oxandrolone (0, 1, 5, 10, 15  $\mu$ g/ml). After 24 h of treatment, the levels of alkaline phosphatase activity were determined using a commercial kit (Pierce Biotechnology, Inc., Rockford, IL). The cells were washed with cold PBS and subjected to three freeze-thaw cycles. These samples were assayed for enzymatic activity with p-nitrophenyl phosphate as a substrate. Sample absorbance was measured at 400 nm with microplate reader. Results were expressed in ALP (OD)/Protein (OD).

*Immunoquantitative assay for collagen type I and osteocalcin:* In order to perform the immunoquantitative assay for type I collagen (4) and osteocalcin, human osteoblastic cells were subcultured on 96 wells plates until they achieved 70% confluence. Oxandrolone was used to stimulate the cells at the concentration of 0, 1, 10, 15 and 30  $\mu$ g/ml for 24 h. Cells were harvested and washed with cold phosphate buffered saline (PBS), fixed in methanol for 10 min at minus 20° C and washed with PBS. They were then pre-incubated with primary antibody for type I collagen and osteocalcin (Santa Cruz Biotechnology Inc., CA), respectively, overnight at 4° C, followed by secondary biotinylated IgG for 30 min at room temperature. After washing with PBS, cells were exposed to p-nitrophenyl phosphate containing levamisole to inhibit the

generation of *p*-nitrophenol by endogenous alkaline phosphatase for 8 min. Sample absorbance was measured at 400 nm with an ELISA reader. The values (OD) were normalized with protein concentration (OD).

*Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on cultured human osteocytic cells:* Total RNA was isolated from osteocytic cultures and gene expression characterized by qRT-PCR analysis using human primers from Qiagen (Valencia, CA) specific for AR and the osteoblast marker proteins alkaline phosphatase, type I collagen, osteocalcin and osteoprotegerin. The qRT-PCR analysis was performed with the iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) on DNase-treated total RNA. Relative expression of the RT-PCR product was determined using the comparative  $\Delta\Delta C_t$  method after normalizing expression with fluorescence to the specific RNA binding dye RiboGreen (Molecular Probes, Eugene, OR, USA) as previously described (10). Real-time qRT-PCR efficiency was determined for each primer set using a five-fold dilution series of total RNA and did not differ significantly from 100%. Individual reaction kinetics was also analyzed to ensure each real-time RT-PCR did not differ significantly from 100% efficiency. Following PCR, specificity of the PCR reaction was confirmed with inverse derivative melt curve analysis. Data is presented as mean  $\pm$  SEM.

*Statistical analyses:* Data are reported as mean  $\pm$  SEM. The statistical significance of intergroup differences was tested using the student *t*-test when the variances were equal.  $P < 0.05$  was considered significant.

## Results

### *Oxandrolone treatment in proliferating human osteoblastic cells*

Proliferating cultures of normal human osteoblastic cells were treated for 24 h with 30 µg/ml oxandrolone or vehicle to determine the acute response to oxandrolone treatment. Although the affinity for oxandrolone for AR is ~100 fold lower than testosterone, oxandrolone treatment still results in AR transactivation (11). In order to characterize the ability of oxandrolone to stimulate translocation of the AR to the nucleus, confocal laser scanning microscopy was employed. When control cultures were stained for AR, diffuse cytoplasm staining but only weak nuclear staining were observed on osteoblast non-stimulated with oxandrolone (Fig. 1A). After stimulation with oxandrolone, increased fluorescence of AR in the nuclei was seen (Fig. 1B), suggesting transactivation by oxandrolone at these concentrations.

We next assessed the effect of oxandrolone treatment on type I collagen, the major constituent of the bone matrix. Immunohistochemistry for collagen type I on cultured osteoblast cells demonstrated an increase in collagen that was dose dependent. When compared to control group (Fig. 1C), a significant increased intensity of fluorescence was seen for cells stimulated with oxandrolone. This was observed when the concentration of oxandrolone was 15 µg/ml (Fig. 1F) and 30 µg/ml (Fig. 1D).

To determine the effect of oxandrolone on additional markers of osteoblast activity, osteoblastic cultures were treated with or without oxandrolone. Alkaline phosphatase activity was determined in cells treated with 30 µg/ml oxandrolone for 24 h (Fig. 2), and there was an increase (7-20%) in activity in a dose-dependent manner. The differences were statistically significant ( $P < 0.05$ ) but the changes were minor compared to the control group. We also performed immunoquantitative assays for type I collagen and osteocalcin on osteoblastic cells.

In cultures treated with oxandrolone (10 or 15  $\mu\text{g/ml}$ ) collagen was increased 21%-35% (Fig. 3;  $P < 0.05$ ). There were no differences in the groups treated with low concentration of oxandrolone (1 or 5  $\mu\text{g/ml}$ ) compared to control group. Thus, after multiple repetitions of the experiment, the data confirmed the results observed by using immunofluorescence.

We next assessed the levels of osteocalcin, an important biochemical indicator of bone turnover (25). Cells treated with oxandrolone of 10, 15 and 30  $\mu\text{g/ml}$  produced a greater level of osteocalcin (11-18%) compared to the control group. The differences were statistically significant ( $P < 0.05$ ) but again the increase was small. Thus, the production of osteocalcin mirrored the pattern of alkaline phosphatase activity.

AR concentrations increase as osteoblasts differentiation, reaching their highest levels in osteocytic cultures (12). Furthermore, the most abundant cell in bone is the osteocytic cell. We therefore determined the effect of oxandrolone treatment on gene expression in mature osteocytic cultures. Normal human osteoblasts were cultured for 10 d, then switched to differentiation medium containing ascorbic acid and  $\beta$ -glycerol phosphate. After 7 d, osteocytic cells were treated with 15  $\mu\text{g/ml}$  oxandrolone. Total RNA was isolated after 24 h or 5 d treatment, and gene expression characterized by qRT-PCR analysis using human primers specific for AR, type I collagen, alkaline phosphatase, osteocalcin and osteoprotegerin (Fig. 5). Although there was little effect of oxandrolone after 24 h of treatment in osteocytic cells on type I collagen, alkaline phosphatase, osteocalcin or osteoprotegerin mRNA abundance, a non-significant increase in AR mRNA was noted. This is consistent with other reports documenting an increase in AR after androgen treatment in osteoblasts (13). After stimulation with oxandrolone for 5 days, a significant decrease in expression of type I collagen was seen ( $P < 0.01$ ). Thus, longer treatments with oxandrolone in osteocytic cultures reduced collagen expression. This data is

consistent with dose-response studies performed after 5 d of treatment with increasing concentrations of oxandrolone (Fig. 6), where AR mRNA is modestly increased but collagen levels are significantly decreased in a dose-dependent fashion with oxandrolone treatment.

## Discussion

In this study we have shown that stimulation of cultured human osteoblasts by oxandrolone results in immunofluorescent detection of AR in the nucleus and increased osteomarkers in these osteoblasts. These data suggest that oxandrolone directly targets human osteoblasts by means of the AR, resulting in increased expression of osteoblast differentiation markers after short-term treatment. Therefore, oxandrolone may act directly on the osteoblast in addition to effects that result in increasing skeletal loading.

Immunohistochemistry and confocal laser scanning microscopy (CLSM) were used to evaluate the expression of AR and type I collagen in osteoblasts, with several advantages over regular fluorescence microscopy (14, 15). While in conventional wide field fluorescence microscopy the emitted light coming from regions above and below the focal plane is collected by the objective lens and contributes to an out-of-focus blur in the final image, in the CLSM a diaphragm rejects the out-of-focus information (14, 15). Therefore, tissue thickness does not interfere with the resulting fluorescence. The increased expression of type I collagen by osteoblasts was also determined by immunoassay confirming the results observed with immunohistochemistry.

The AR is a steroid receptor that generally mediates biologic responses to androgens. The increased expression of AR in the present study is consistent with that documented in the literature (13). In bone tissue the AR is expressed in a variety of cell types, but its specific role in maintenance of skeletal homeostasis remains controversial (16). In an experimental study, androgen deficiency was shown to result in a substantial loss of cancellous bone in the axial and appendicular skeleton of aged male rats and that this osteopenia is associated with a sustained increase in bone turnover (17). Consistent with this, the bone phenotype that develops in a global

AR null (ARKO) male mouse model is a high-turnover osteopenia, with reduced trabecular bone volume and a significant stimulatory effect on osteoclast function (18-19).

Alkaline phosphatase is well recognized as a marker that reflects osteoblastic activity (20). Kasperk et al reported that androgens increase the alkaline phosphatase activity in osteoblast culture (21-23). However, there are also reports of androgens either inhibiting (24) or having no effect on alkaline phosphatase activity (25, 26), which may reflect both the complexity of osteoblastic differentiation and the various model systems employed. In a clinical study, Murphy et al (1) have shown that oxandrolone administration increases levels of serum alkaline phosphatase in treated patients by 6 months versus controls. In the present study, the cells treated with oxandrolone produced a greater level of alkaline phosphatase. The elevated activity of this cellular enzyme suggests an early increase in the osteoblast differentiation process.

Osteocalcin appears to be a bone-specific gla-containing protein, accounting for 10-20% of the non-collagenous protein in bone. While the *in vivo* function of osteocalcin remains unclear, its affinity for bone mineral constituents implies a role in bone formation. Hence it has been shown that osteocalcin is a biochemical indicator of bone turnover (27). In our study, proliferating cells treated for 24 h with oxandrolone produced small but statistically significant increases in osteocalcin production compared to control group.

In order to test the effect of oxandrolone on osteocyte-like cells differentiated from osteoblasts at molecular level, the human osteoblastic cells were cultured in differentiation medium and then exposed to oxandrolone. The results showed that short-term treatment produced little effect on type I collagen, alkaline phosphatase, osteocalcin, osteoprotegerin, or AR mRNA. Long-term treatment decreased type I collagen expression, consistent with decreased collagen expression observed by Wiren et al (16) in AR-transgenic mice with targeted

AR overexpression in the osteoblast lineage (16). The results described here are consistent with observations reported *in vivo* with male AR-transgenic mice, where calvarial thickening is observed and cortical formation is altered with surface periosteal expansion but inhibition of inner endosteal deposition, consistent with the known effects of androgen to stimulate periosteal apposition. The dramatic inhibition at the endosteal envelope may be responsible for a modest decrease in cortical bone area and reductions in biomechanical properties observed (16). Thus, taken all data together, our results suggest that osteoblast cells are targeted by androgens to transactivate AR in bone.

Murphy et al (1) have recently shown that oxandrolone administration increases lean body mass three to six months before an increase in bone mineral content is observed. On the other hand, we have found an increase in collagen production when high doses of oxandrolone were used to stimulate osteoblasts. The applicability of our findings in this study to the *in vivo* effects of oxandrolone in burned children (1) is not clear. While an *in vitro* environment may not adequately reproduce the *in vivo* situation, many potential variables are eliminated during *in vitro* studies, and it is possible to investigate a specific effect of a drug on cells. The time frame of *in vivo* and *in vitro* settings is plainly different, and the concentration of oxandrolone used to stimulate cells in the *in vitro* setting is probably higher than the one used in the clinical trial (1). Therefore, the results may not be strictly comparable. Although this study demonstrates that oxandrolone is capable of affecting the osteoblast AR and stimulating type I collagen synthesis, regulatory mechanisms that are present only in a complex *in vivo* situation may account for increased collagen turnover or degradation after synthesis.

Moreover, the delay in the increase of total body bone mineral content reported in the clinical study by Murphy et al (1) may be explained in part by the acute inhibition of bone

formation and osteoblast differentiation after a severe burn as previously reported (3,4). Thus there may have been a lack of osteoblasts and therefore, osteoblast AR to mediate a response to oxandrolone.

In conclusion, the use of high-dose oxandrolone results in increased nuclear fluorescence of the osteoblast AR, increased osteoblast differentiation markers in cultured osteoblasts and decreased bone marker in cultured osteocyte-like cells *in vitro*. Oxandrolone may have the ability to directly stimulate bone collagen synthesis, over and above its effect on skeletal loading effected through an increase in lean body mass following long-term treatment. However, longer exposure may no longer be directly anabolic in mature bone cells. This observation may help to explain the variability and confusion regarding androgen actions on the skeleton.

## **Acknowledgments**

This project was funded in part by NIH 1 P50 GM 60338 and presented in part at the 26th Annual Meeting of the American Society for Bone and Mineral Research, Seattle WA, 1-5 October 2004.

## References

1. Murphy KD, Thomas S, Mlcak RP, Chinkes DC, Klein GL, Herndon DN (2004) "Effects of long-term oxandrolone administration on severely burned children" *Surgery* 136: 219-24.
2. Hart DW, Herndon DN, Klein G, Lee SB, Celis M, Mohan S, Chinkes DL, Wolf SE. (2001) "Attenuation of post-traumatic muscle catabolism and osteopenia by long-term growth hormone therapy" *Jun*;233(6):827-34
3. Klein GL, Herndon DN, Goodman WG, Langman CB, Phillips WA, Dickson IR, Eastell R, Naylor KE, Maloney NA, Desai M, Benjamin D and Alfrey AC (1995) "Histomorphometric and biochemical characterization of bone following acute severe burns in children" *Bone* 17: 455-60
4. Klein GL, Bi LX, Sherrard DJ, Beavan SR, Ireland D, Compston JE, Williams WG, Herndon DN. (2004) "Evidence supporting a role of glucocorticoids in short-term bone loss in burned children" *Osteoporos Int* 15: 468-74
5. Nakano Y, Morimoto I, Ishida O et al The receptor, metabolism and effects of androgen in osteoblastic MC3T3 cells *Bone Miner* 1994; 26: 245-59.
6. Braidman IP, Baris C, Selby PL, Adams JE, Freemont AJ, Hoyland JA (2000) "Preliminary report of impaired oestrogen receptor-alpha expression in bone, but no involvement of androgen receptor, in male idiopathic osteoporosis", *J Pathol* 192:90-6.
7. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS (1990) "Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with

- osteoblast proliferation and differentiation during formation of the bone extracellular matrix” *J Cell Physiol* Jun;143(3):420-30
8. Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ (1992) “Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development” *J Bone Miner Res.* Jun;7(6):683-92
  9. Birnbaum RS, Wren KM (1994) “Changes in insulin-like growth factor-binding protein expression and secretion during the proliferation, differentiation, and mineralization of primary cultures of rat osteoblasts” *Endocrinology.* Jul;135(1):223-30
  10. Hashimoto JG, Beadles-Bohling AS, Wren KM (2004) “Comparison of RiboGreen and 18S rRNA quantitation for normalizing real-time RT-PCR expression analysis” *Biotechniques* Jan;36(1):54-6, 58-60
  11. Kemppainen JA, Langley E, Wong CI, Bobseine K, Kelce WR, Wilson EM. (1999) “Distinguishing androgen receptor agonists and antagonists: distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone” *Mol Endocrinol* Mar;13(3):440-54
  12. Wren KM, Chapman Evans A, Zhang XW (2002) “Osteoblast differentiation influences androgen and estrogen receptor-alpha and -beta expression” *J Endocrinol* Dec;175(3):683-94
  13. Wren K, Keenan E, Zhang X, Ramsey B, Orwoll E (1999) “Homologous androgen receptor up-regulation in osteoblastic cells may be associated with enhanced functional androgen responsiveness” *Endocrinology* Jul;140(7):3114-24

14. Buttino I, Ianora A, Carotenuto Y, Zupo V, Miralto A (2003) "Use of the confocal laser scanning microscope in studies on the developmental biology of marine crustaceans" *Microsc Res Tech* 2003; 60: 458-64
15. Diaspro A editor. *Confocal and two-photon microscopy. Foundations, applications and advances*, 2002 New York; Wiley-Liss Inc, p 1-567.
16. Wiren KM, Zhang XW, Toombs AR, Kasparcova V, Gentile MA, Harada S, Jepsen KJ (2004) "Targeted overexpression of androgen receptor in osteoblasts: unexpected complex bone phenotype in growing animals" *Endocrinology* 145: 3507-22
17. Erben RG, Eberle J, Stahr K, Goldberg M (2000) "Androgen deficiency induces high turnover osteopenia in aged male rats: a sequential histomorphometric study" *J Bone Miner Res* 15: 1085-98.
18. Worldarski KH, Reddi AH (1986) "Alkaline phosphatase as a marker of osteoinductive cells" *Calcif Tissue Int* 39:382-385.
19. Yeh S, Tsai M, Xu Q, Mu X, Lardy H, Huang K, Lin H, Yeh S, Altuwaijri S, Zhou X, Xing L, Boyce B, Hung M, Zhang S, Gan L, Chang C, Hung M 2002 Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. *Proc Natl Acad Sci USA* 99:13498-13503
20. Kawano H, Sato T, Yamada T, Matsumoto T, Sekine K, Watanabe T, Nakamura T, Fukuda T, Yoshimura K, Yoshizawa T, Aihara K, Yamamoto Y, Nakamichi Y, Metzger D, Chambon P, Nakamura K, Kawaguchi H, Kato S 2003 Suppressive function of androgen receptor in bone resorption. *Proc Natl Acad Sci USA* 100:9416-9421

21. Kasperk CH, Wergedal JE, Farley JR, Linkhart TA, Turner RT, Baylink DJ (1989)  
“Androgens directly stimulate proliferation of bone cells in vitro” 124:1576-1578
22. Kasperk CH, Faehling K, Börcsök I, Ziegler R(1996) “Effects of androgens on  
subpopulations of the human osteosarcoma cell line SaOS2” *Calcif Tissue Int.* 58:376-  
382.
23. Kasperk CH, Wakley GK, Hierl T, Ziegler R (1997) “Gonadal and adrenal androgen are  
potent regulator of human bone cell metabolism in vitro” *J Bone Miner Res.* 12:464-471.
24. Hofbauer L, Hicok K, Khosla S 1998 Effects of gonadal and adrenal androgens in a novel  
androgen-responsive human osteoblastic cell line. *J Cell Biochem* 71:96-108
25. Takeuchi M, Kakushi H, Tohkin M 1994 Androgens directly stimulate mineralization  
and increase androgen receptors in human osteoblast-like osteosarcoma cells. *Biochem  
Biophys Res Commun* 204:905-911
26. Gray C, Colston K, Mackay A, Taylor M, Arnett T 1992 Interaction of androgen and  
1,25-dihydroxyvitamin D3: effects on normal rat bone cells. *J Bone Miner Res* 7:41-46
27. Seibel MJ (2003) “Biochemical markers of bone remodeling” *Endocrinol Metab Clin  
North Am* 32:83-113

## Figure Legends

### **Figure 1. Confocal scanning laser microscopic analysis of human osteoblastic cells after**

**oxandrolone treatment.** A. Confocal scanning laser microscopy depicting AR nuclear fluorescence of human osteoblastic cells not stimulated with oxandrolone. B. Confocal scanning laser microscopy depicting the increased AR nuclear fluorescence of human osteoblastic cells stimulated with oxandrolone 30 µg/ml. C. Confocal scanning laser microscopy depicting cytoplasmic fluorescence of type I collagen in human osteoblastic cells not stimulated with oxandrolone. D. Confocal scanning laser microscopy depicting increased cytoplasmic fluorescence of type I collagen in human osteoblastic cells stimulated with oxandrolone 30 µg/ml. E. Internal negative control for primary antibodies of AR and type I collagen. F. Confocal scanning laser microscopy depicting increased cytoplasmic fluorescence of type I collagen in human osteoblastic cells stimulated with oxandrolone 15 µg/ml. Compare to Figures 1C and 1D.

### **Figure 2. Stimulation of alkaline phosphatase activity by oxandrolone in proliferating**

**human osteoblastic cultures.** Proliferating cultures were treated acutely for 24 h with 0, 1, 5, 10, 15 µg/ml oxandrolone. Data are expressed as mean  $\pm$  SEM of six determinations.

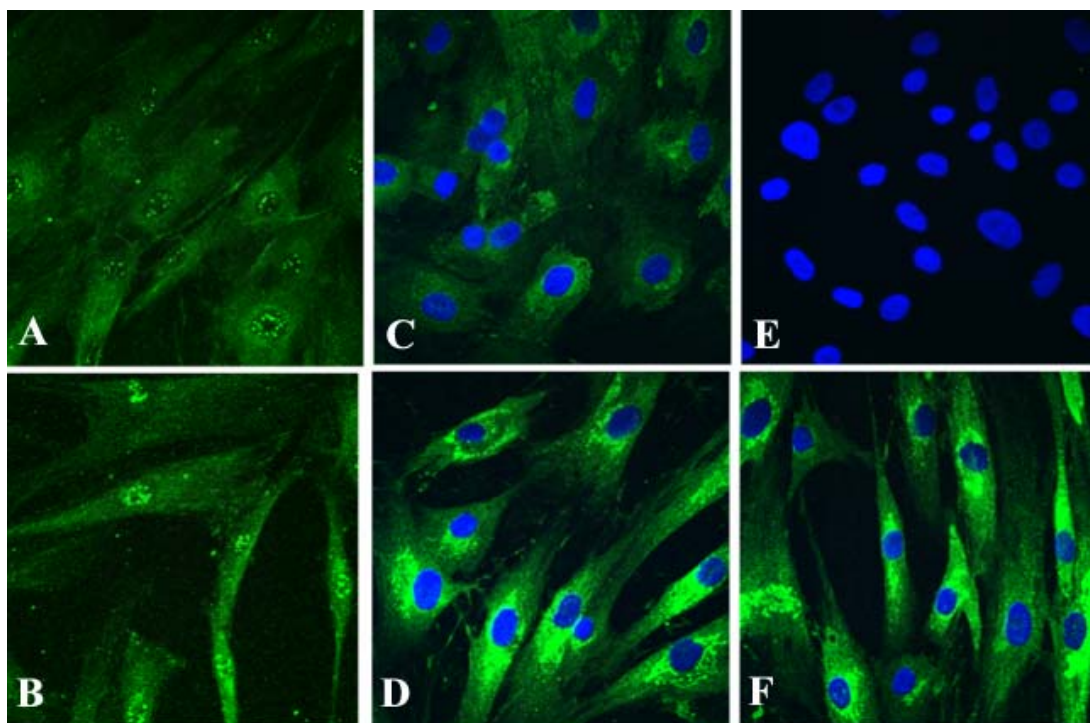
### **Figure 3. Increased osteocalcin levels after after oxandrolone treatment.**

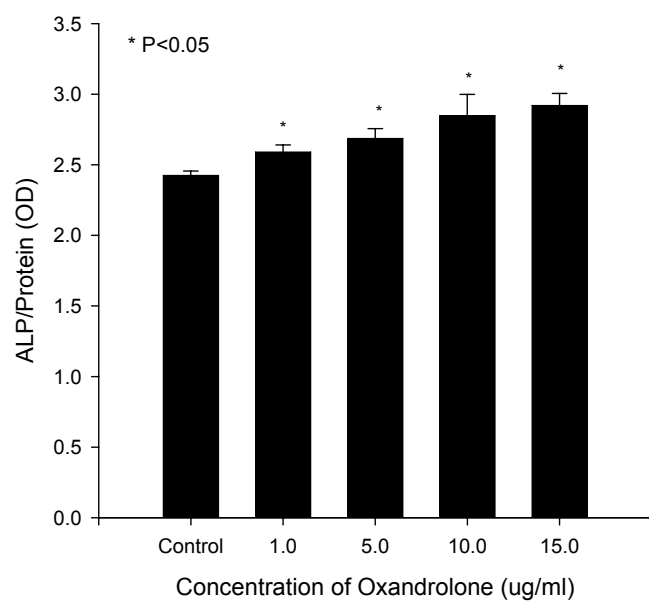
Human osteoblastic cultures were exposed to oxandrolone (0, 1, 5, 10, 15 µg/ml) for 24 h. Data are expressed as mean  $\pm$  SEM of six determinations.

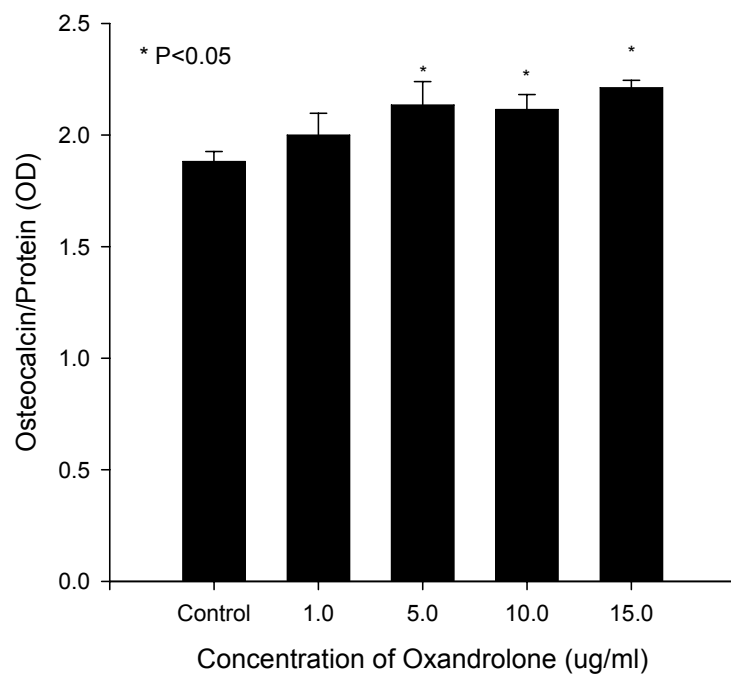
**Figure 4. Enhanced type I collagen secretion after oxandrolone exposure.** Proliferating cultures were treated with 0, 1, 5, 10, 15 ug/ml oxandrolone for 24 h. Data are expressed as mean  $\pm$  SEM of six determinations.

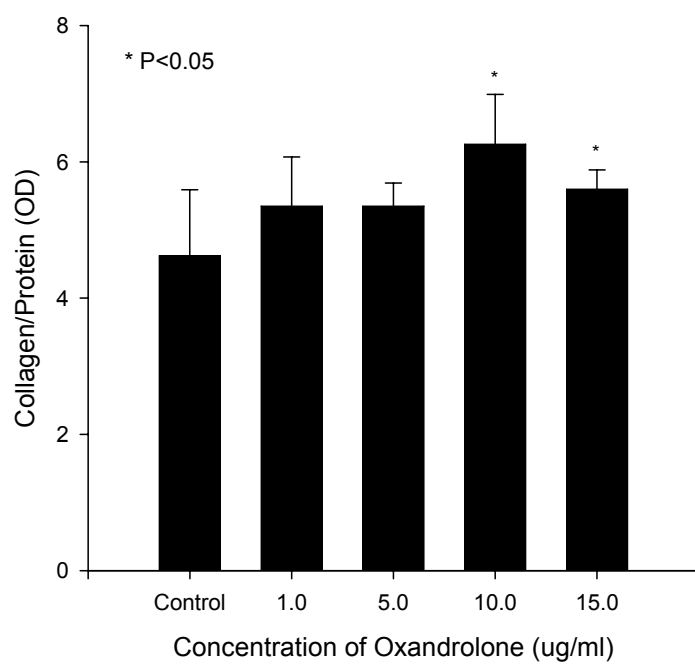
**Figure 5. Consequence of oxandrolone treatment on gene expression in normal human osteocytes: time course analysis.** Normal human osteoblastic cells were cultured for 10 d to confluence, then switched to differentiation medium containing ascorbic acid and  $\beta$ -glycerol phosphate. After 7 d, osteocytic cells were treated with 15  $\mu$ g/ml oxandrolone. Total RNA was isolated after 24 h or 5 d treatment, and gene expression characterized by qRT-PCR analysis using human primers specific for AR, type I collagen (col), alkaline phosphatase (ALP), and osteocalcin (OC) and osteoprotegerin (OPG). n=3-4. \*\*,  $P < 0.01$ .

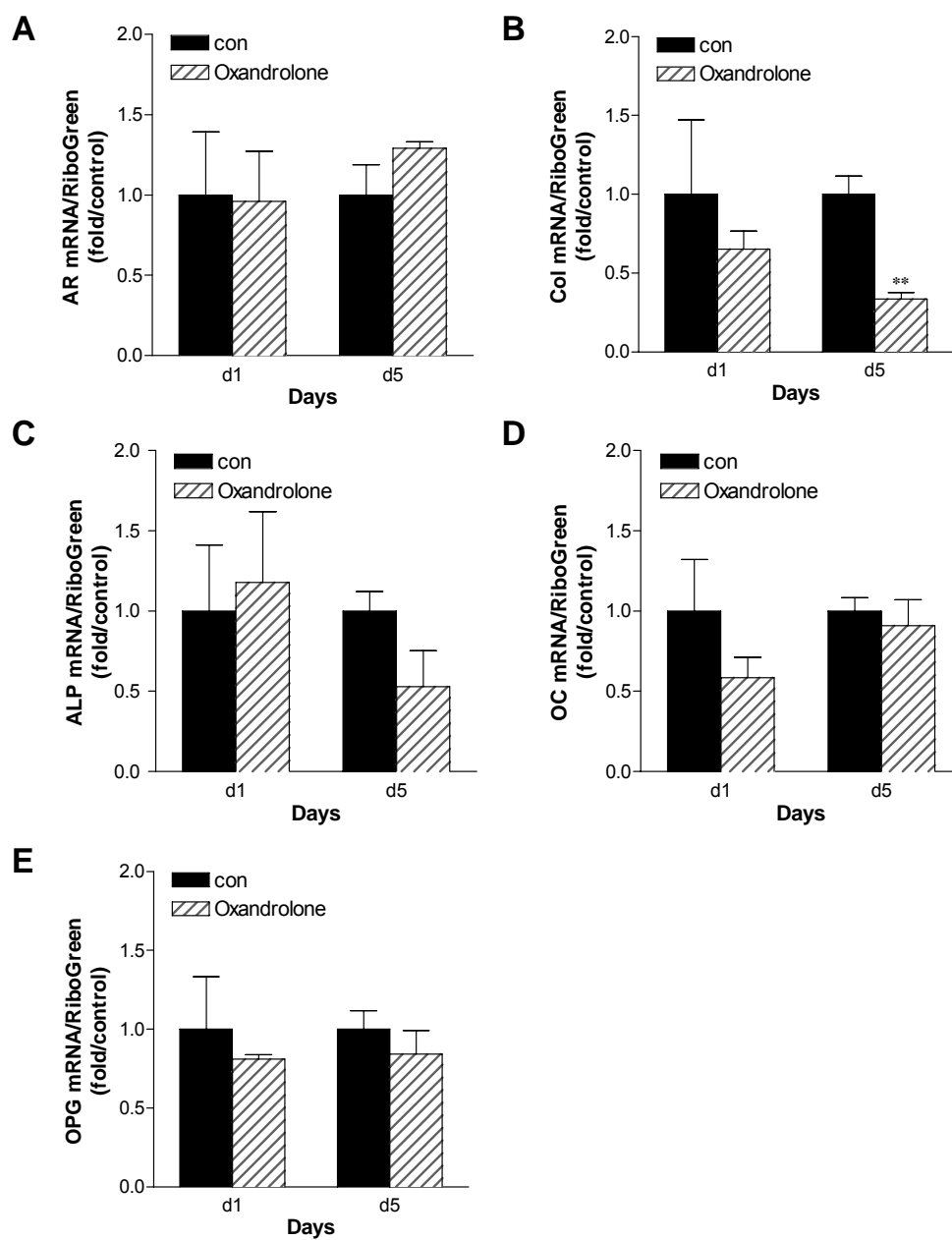
**Figure 6. Consequence of oxandrolone treatment on gene expression in normal human osteocytes: dose response analysis.** Normal human osteoblastic cells were cultured as described in Figure 5. Osteocytic cultures were treated for 5 d with 0, 1, 5, 10 or 15  $\mu$ g/ml oxandrolone. Total RNA was isolated and gene expression characterized by qRT-PCR analysis using human primers specific for AR, type I collagen (col), alkaline phosphatase (ALP), osteocalcin (OC) and osteoprotegerin (OPG). n=2-4. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

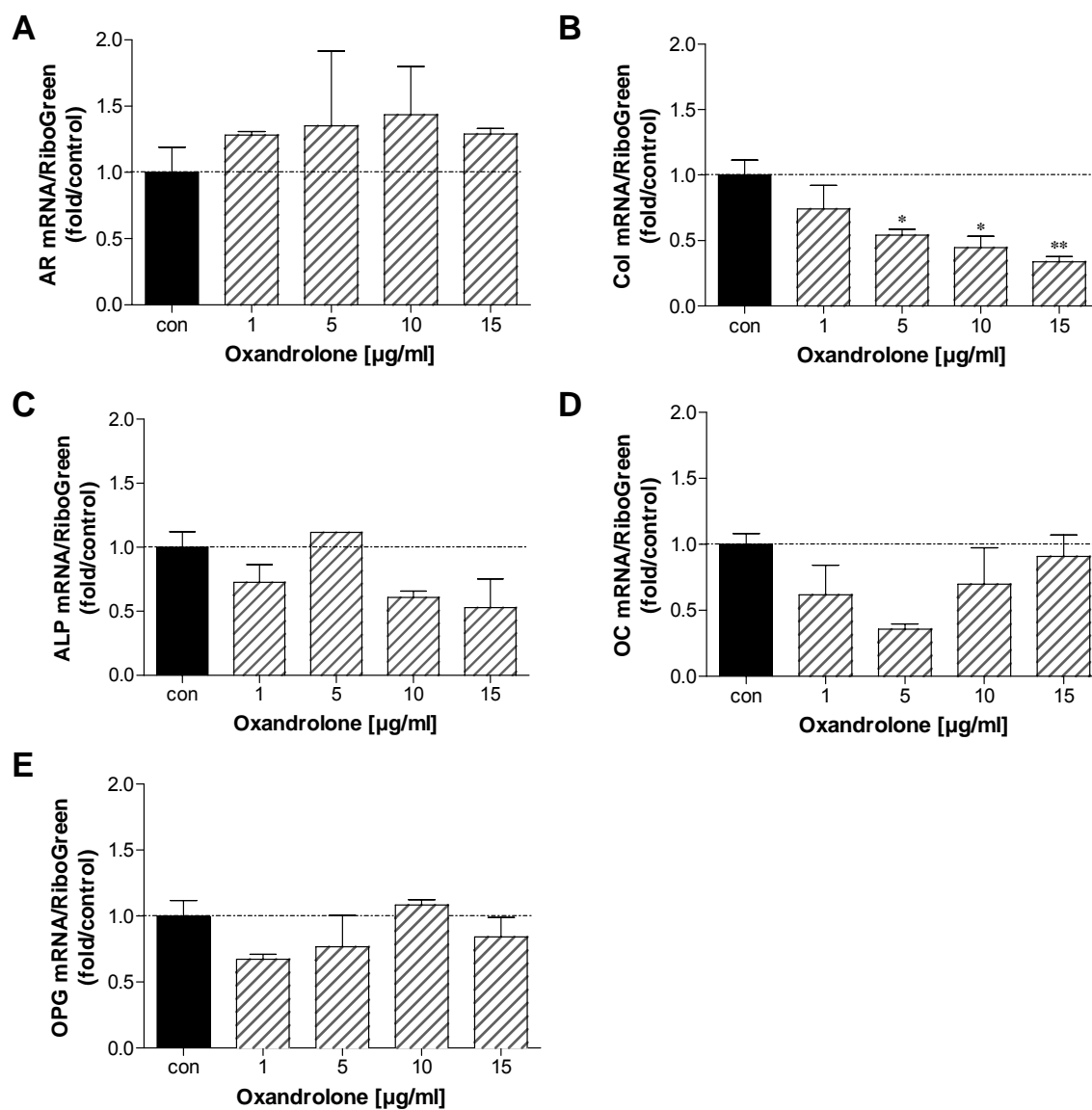
**Figure 1:**

**Figure 2:**

**Figure 3:**

**Figure 4:**

**Figure 5**

**Figure 6**

### Appendix 3:

Wiren KM: Direct androgen action in the skeleton. (Wellness/Fitness Research Abstract #121)  
PRMRP Military Health Research Forum, 2006

## **DIRECT ANDROGEN ACTION IN THE SKELETON** **Kristine Wiren, Ph.D.** **Oregon Health & Science University and VA Medical Center**

**BACKGROUND:** Androgens have been shown to be important mediators of bone growth and remodeling independent of estrogen, and have been used for the treatment of osteoporosis. Our previous characterization of transgenic mice with androgen receptor (AR) overexpression in the osteoblast lineage under control of type I collagen promoter (col3.6 AR-transgenic mice) demonstrated proof of principle for the specific importance of androgen and AR directly in bone. Since bone architecture and bone material properties play important roles in stress fracture, analysis of AR-transgenic mice represents a unique opportunity to characterize the consequences of androgen action in both genders on bone microarchitectural quality and the integrity of the skeleton *in vivo*. A better understanding of the consequences of androgen action in bone is also important given increased anabolic steroid abuse.

**PURPOSE:** To better understand the role of androgen signaling in bone, we have genetically engineered a second set of transgenic mice in which AR overexpression is skeletally targeted to mature osteoblasts (col2.3) to contrast with col3.6 (stromal cells and throughout the osteoblast lineage). The central hypothesis is that AR transactivation in the osteoblast lineage provides key regulatory signals that regulate the progression of osteoblast differentiation and osteogenesis, control the resorption of calcified bone, and modulate lineage determination in the marrow, to influence skeletal architecture and matrix quality.

**METHODS:** Two distinct lines of AR-transgenic mice (col2.3 and col3.6) have been created with bone-targeted AR overexpression to provide models for the characterization of enhanced androgen signaling in distinct skeletal compartments. Characterization of col2.3 AR-transgenic mice included body weight and length gain over 6 months, hormone measurements and gene expression analyses using real-time qRT-PCR. Bone quality of whole femur was assessed at 2 months of age in four-point bending to measure stiffness, maximum load, post-yield deflection (a measure of ductility), and work-to-failure. Femurs were also evaluated for morphology using microComputed Tomography ( $\mu$ CT). Because of distinct and overlapping expression profiles, comparison of the skeletal phenotypes characterized in these two models is postulated to aid in the identification of cells within the osteoblast lineage that are most important in mediating a specific response in bone modeling/remodeling characteristics.

**RESULTS:** There were no differences in serum levels of testosterone,  $17\beta$ -estradiol, in body weight or length gain in col2.3 AR-transgenic mice, in contrast to a significant reduction in body weight in male col3.6 AR-transgenic mice. Similar to col3.6 mice, serum osteocalcin was reduced and osteoprotegerin was increased only in male col2.3 mice. Col2.3 AR-transgene expression was elevated in bone (calvaria and tibia) but 100-3,000 fold lower in other tissues surveyed including heart, fat, spleen, kidney, liver etc, demonstrating the expected profile of skeletal targeting. Importantly, the level of AR-transgene overexpression in calvaria was similar between col2.3 and col3.6 mice (~15-20-fold vs. endogenous AR). Calvarial thickening was not observed in col2.3 males. Male col2.3 AR-transgenic mice showed only an 8-9% reduction in stiffness and body-weight adjusted maximum load (whole bone strength) compared to controls, whereas the col3.6 AR-transgenic mice showed 33-36% reductions compared to controls. Males of both AR-transgenic lines showed a similar reduction in bone ductility (increased brittleness) assessed with post-yield deflection (48% for col3.6 and 31% for col2.3), and this in

part leads to a reduction in work-to-failure (65% for col3.6 and 26% for col2.3). Thus, biomechanical data clearly shows that AR overexpression in stromal cells and throughout the osteoblast lineage (col3.6) has a more dramatic bone phenotype compared to AR overexpression in mature osteoblasts (col2.3). Morphological analysis by  $\mu$ CT demonstrates reduced cortical area arising from an expanded marrow cavity. These changes in bone marrow space resulted in a small reduction in moment of inertia.

**CONCLUSION:** In both models, enhanced androgen action occurs only in those cells with elevated levels of AR (skeletally-targeted) as a consequence of enhanced AR signaling, without changes in circulating steroid levels and without systemic androgen administration. AR transactivation (in young col2.3 vs. col3.6 AR-transgenic males) in mature osteoblasts is primarily responsible for mediating the effects of androgen on matrix quality and/or mineralization, while immature cells mediate effects of androgen on the periosteum and potentially body composition. Further, enhanced androgen signaling during growth of the skeleton appears to produce a low turnover state and may be detrimental to overall bone quality and strength with more damageability. Combined, these results raise concerns regarding androgen administration in the still-growing skeleton.

#### Appendix 4:

Semirale A, Zhang X-W, **Wiren KM**: Androgen receptor overexpression in precursor cells and not mature osteoblasts influences body composition by reducing adiposity and increasing lean mass. (Abstract#SU466 ) J Bone Miner Res, 2006

#### **Androgen Receptor Overexpression in Precursor Cells and Not Mature Osteoblasts Influences Body Composition by Reducing Adiposity and Increasing Lean Mass**

A. Semirale<sup>\*1</sup>, X. Zhang<sup>1</sup> and K. M. Wiren<sup>1</sup>. <sup>1</sup>VA Medical Center and Oregon Health & Science University, Portland, OR, USA.

To examine the consequence of enhanced androgen signaling during skeletal development, two independent transgenic (tg) lines of mice have been generated. Androgen receptor (AR) overexpression controlled by two fragments of the  $\alpha 1(I)$ -collagen promoter (3.6 kb or 2.3 kb) results in distinct phenotypes due to enhanced androgen signaling without systemic androgen treatment. In col3.6 mice, AR is expressed in stromal precursors and throughout the osteoblast lineage while col2.3-driven AR expression is restricted to mature osteoblasts and osteocytes. AR overexpression in col3.6 AR-tg male mice had a dramatic effect on body composition including increased lean mass but reduced percent fat mass as assessed by dual energy X-ray absorptiometry (DXA). White adipose tissue dissection also revealed a decrease in fat depot weight. In addition, col3.6 AR-tg males exhibited a 20-25% reduction in body weight when compared to age-matched, littermate controls. No significant differences were observed in females, presumably due to their low circulating testosterone levels. In contrast, male col2.3 AR-tg mice did not exhibit significant differences in lean or percent fat mass, suggesting that immature precursor cells from the mesenchymal stem cell (MSC) lineage are the likely target of androgen action in col3.6 AR-tg mice. The decrease in body weight and reduced fat mass observed in col3.6 AR-tg males was not a consequence of alterations in baseline locomotor activity assessed by an automated activity monitor. In addition, no obvious difference in food consumption was observed between male wild type and col3.6 AR-tg mice. Histological examination of gonadal fat depots from col3.6 AR-tg mice was performed to assess fat morphology and revealed a ~ 50% reduction in the mean diameter of adipocytes in transgenic males suggesting a functional modification. Combined, these data indicate that AR transactivation in the MSC lineage in col3.6 AR-tg but not col2.3 AR-tg mice provides key regulatory signals that influence body composition to reduce fat quantity and quality. The hypothesis that androgen affects body composition by regulating pluripotent MSC differentiation provides a unifying explanation for the observed discordant changes in bone/muscle vs. fat mass caused by androgen treatment. Further analysis will be important to identify pathways responsible for the deleterious consequences of aging and associated hypogonadism on central fat accumulation, muscle weakness and declining bone mass.

## Appendix 5:

Wiren KM, Zhang X-W, Semirale A, Price C, Woo A, Jepsen KJ: Targeted androgen action in mature vs. preosteoblasts eliminates periosteal stimulation and reduces bone quality during growth. (Abstract#1015 ) J Bone Miner Res, 2006

**\*Selected for Oral Presentation ASBMR**

### **Targeted Androgen Action In Mature vs. Preosteoblasts Eliminates Periosteal Stimulation And Reduces Bone Quality During Growth**

K. M. Wiren<sup>1</sup>, X. Zhang<sup>1</sup>, A. Semirale<sup>1</sup>, C. Price<sup>2</sup>, A. Woo<sup>\*2</sup>, K. J. Jepsen<sup>2</sup>. <sup>1</sup>VA Medical Center, Oregon Health & Science Univ, Portland, OR, USA, <sup>2</sup>Orthopaedics, Mt. Sinai School of Medicine, NY, NY, USA.

Androgen treatment increases bone mass in the hypogondal elderly, but effects during skeletal modeling are poorly understood. To develop an understanding of the cell types important in mediating androgen action, we have constructed and compared two distinct transgenic lines of mice employing different  $\alpha 1(I)$ -collagen promoter fragments to control skeletally-targeted AR overexpression. The col3.6 AR-transgenic (AR-tg) mice demonstrate AR overexpression throughout the osteoblast lineage including the periosteum, while col2.3 AR-tg mice have more restricted overexpression in mature osteoblasts and osteocytes. In both col3.6 and col2.3 AR-tg males at 8-weeks of age, serum osteocalcin was reduced and osteoprotegerin was increased. Calvarial thickening was observed in col3.6 but not in col2.3 AR-tg males. Femur length was reduced 11% in male col3.6 vs. only 2% in col2.3 AR-tg males. Morphological analysis in col2.3 males by  $\mu$ CT demonstrated reduced mineral accumulation ( $P < 0.0001$ ) and in contrast to col3.6 mice, no effect on periosteal apposition but reduced cortical area arising from an 11% expansion of the marrow cavity. Both col2.3 AR-tg mice and col3.6 mice demonstrated a reduction in moment of inertia (10% vs. 16%). Mechanical testing showed a 10-12% reduction in stiffness and adjusted maximum load (whole bone strength) in male col2.3 AR-tg mice compared to controls, whereas the col3.6 AR-tg mice showed 25-31% reductions vs. controls. Males of both AR-tg lines showed reductions in bone ductility (brittleness/matrix quality) assessed with post-yield deflection (51% for col3.6; 30% for col2.3), which in part leads to similar reductions in work-to-failure (47% for col3.6; 31% for col2.3). Thus, androgen action in stromal cells and throughout the osteoblast lineage (col3.6) results in a more dramatic bone phenotype compared to AR overexpression in mature osteoblasts (col2.3). In both models, enhanced androgen action occurs only in those cells with elevated levels of AR without changes in circulating steroid levels. We conclude AR transactivation in mature osteoblasts is primarily responsible for mediating effects of androgen on turnover, matrix quality and/or mineralization, while immature cells mediate effects on the periosteum and body composition. Androgen signaling in osteoblasts during growth may produce a low turnover state, and the consequences are detrimental to overall matrix quality and bone strength. Combined, these results raise concerns regarding androgen administration or anabolic steroid misuse/abuse in the still-growing skeleton.

**Author Disclosure Block:** K.M. Wiren, None.